Vol. 32, No. 4, December 1955

Illinois U Library

The Journal of Experimental Biology

EDITED BY

V. B. WIGGLESWORTH and J. A. RAMSAY

Contents

PAGE

J. A. C. Nicol. Nervous regulation of luminescence in the sea pansy *Renilla köllikeri* . 619

G. A. Horridge. The nerves and muscles of medusae. III. A decrease in the refractory period following repeated stimulation of the muscle of *Rhizostoma pulmo* 636

G. A. Horridge. The nerves and muscles of medusae. IV. Inhibition in *Aequorea forskalea* 642

V. B. Wigglesworth. The role of the haemocytes in the growth and moulting of an insect, *Rhodnius prolixus* (Hemiptera) 649

J. Shaw. Ionic regulation in the muscle fibres of *Carcinus maenas*. II. The effect of reduced blood concentration 664

John Buck and Margaret Keister. Further studies of gas-filling in the insect tracheal system 681

Ellen Thomsen and Kirsten Hamburger. Oxygen consumption of castrated females of the blow-fly, *Calliphora erythrocephala* Meig. 692

G. G. Selman and C. H. Waddington. The mechanism of cell division in the cleavage of the newt's egg. (With Plates 1 and 2) 700

J. M. Mitchison and M. M. Swann. The mechanical properties of the cell surface. III. The sea-urchin egg from fertilization to cleavage 734

D. R. Swift. Seasonal variations in the growth rate, thyroid gland activity and food reserves of brown trout (*Salmo trutta* Linn.) 751

Betty I. Roots. The water relations of earthworms. I. The activity of the nephridiostome cilia of *Lumbricus terrestris* L. and *Allolobophora chlorotica* Savigny, in relation to the concentration of the bathing medium 765

J. Gray. The movement of sea-urchin spermatozoa. (With Plates 3 and 4) 775

J Gray and G. J. Hancock. The propulsion of sea-urchin spermatozoa 802

D M. Ross. Facilitation in sea anemones. IV. The quick response of *Calliactis parasitica* at high temperatures 815

J. A. Ramsay, R. H. J. Brown and P. C. Croghan. Electrometric titration of chloride in small volumes 822

Index of subjects 830

Index of authors 833

Published for The Company of Biologists Limited
CAMBRIDGE UNIVERSITY PRESS
CAMBRIDGE, AND BENTLEY HOUSE, LONDON

AGENT FOR U.S.A.
ACADEMIC PRESS INC.,
125, EAST 23RD STREET, NEW YORK, U.S.A.

Price 30s. net *Subscription per volume 84s. net*

THE JOURNAL OF PHYSIOLOGY

NOVEMBER 1955. VOL. 130, NO. 2. 25*s.*

Linzell, J. L. Some observations on the contractile tissue of the mammary glands.

Anderson, J. A method for estimating Tm for phosphate in man.

Whaler, B. C. The metabolism of amino acids by the small intestine.

Coombs, J. S., Eccles, J. C. and Fatt, P. The electrical properties of the motoneurone membrane.

Coombs, J. S., Eccles, J. C. and Fatt, P. The specific ionic conductances and the ionic movements across the motoneuronal membrane that produce the inhibitory post-synaptic potential.

Coombs, J. S., Eccles, J. C. and Fatt, P. Excitatory synaptic action in motoneurones.

Coombs, J. S., Eccles, J. C. and Fatt, P. The inhibitory suppression of reflex discharges from motoneurones.

Howell, F. R. and Richards, T. G. The determination of pressure-volume changes in the femoral tree of the cat in relation to vascular tonus and the resistance offered to blood flow.

Conway, E. J., Geoghegan, Honor and McCormack, J. I. Autolytic changes at zero centigrade in ground mammalian tissues.

Conway, E. J. and Geoghegan, Honor. Molecular concentration of kidney cortex slices.

Florey, E. and McLennan, H. Effects of an inhibitory factor (Factor I) from brain on central synaptic transmission.

Edwards, Charles and Larrabee, Martin G. Effects of anaesthetics on metabolism and on transmission in sympathetic ganglia of rats. Measurement of glucose in microgram quantities using glucose oxidase.

Beaconsfield, P. and Ginsburg, Jean. The effect of body posture on the hand blood flow.

Andrew, B. L. The respiratory displacement of the larynx: a study of the innervation of accessory respiratory muscles.

Ritchie, J. M. and Wilkie, D. R. The effect of previous stimulation on the active state of muscle.

Proceedings.

Subscription price from Vol. 131, 80s. net per volume of 3 parts

CAMBRIDGE UNIVERSITY PRESS
BENTLEY HOUSE, 200 EUSTON ROAD, LONDON, N.W.1

THE NINTH SYMPOSIUM
OF THE SOCIETY FOR
EXPERIMENTAL BIOLOGY

Fibrous Proteins
and their
Biological
Significance

EDITED BY
R. BROWN & J. F. DANIELLI

Being 22 papers read at the Symposium of the Society for Experimental Biology held at Leeds in September 1954.

50*s. net*

CAMBRIDGE
UNIVERSITY PRESS

VOLUME 32, No. 4 DECEMBER 1955

NERVOUS REGULATION OF LUMINESCENCE IN THE SEA PANSY *RENILLA KÖLLIKERI*

BY J. A. C. NICOL*
The Marine Biological Laboratory, Plymouth

(*Received 2 December* 1954)

INTRODUCTION

Recent investigations of the sea pansy *Renilla*, carried out independently by Buck and myself, have shown that this animal is particularly favourable for the study of luminescent responses. Under tactile and electrical stimulation the sea pansy produces distinct luminous waves which run over the entire surface of the rachis from the point of stimulation. Luminescence is under the control of a diffuse, unpolarized nerve net, and the course and speed of the luminescent wave are indices of direction and velocity of propagation of excitation in the nerve net.

Luminescence in *Renilla* is intracellular, and the light appears from evenly distributed points all over the upper surface of the rachis. Buck (1955) has pointed out that the point-spots of light arise from three loci, viz. the siphonozooids, and the body and tentacles of the autozooids. The luminous wave which sweeps over the colony results from the glowing of successive ranks of zooids (Parker, 1919, 1920).

In correspondence with results obtained on neuro-effector functioning in other coelenterates, it has been found that the luminescent responses of *Renilla* display marked facilitation. From various lines of evidence it has been deduced that facilitatory processes occur terminally, in the region of the photocytes. In contrast, through-conduction (1:1 transmission) appears to be the normal condition in the nerve net.

Each luminescent wave, including the first, invades the whole rachis in untreated fresh animals. However, evidence for interneural facilitation under special conditions has been secured by partially transecting animals, and studying transmission across the tissue junctions so produced. By this means it has been possible to produce preparations in which additional stimuli are required to bridge the junction between the partially separated segments of the animal. Such junctions are also subject to fatigue. Interneural facilitation, thus artificially revealed, provides functional evidence for synapses and multiplicity of conducting neurones in the nerve net (Buck, 1955; Buck & Coyle, 1955; Nicol, 1955).

The present paper presents further information about the luminescent responses of the pennatulid *R. köllikeri*. Neuro-effector functioning is analysed in more detail, and several additional aspects of the luminescent response have been explored by more refined methods, especially transmission velocity and response parameters.

---

* Guggenheim Canadian Fellow, 1953.

depending on its previous history and level of excitability. Augmentation of response in successive bursts is illustrated in Fig. 4. The effect has been observed for as many as 10 successive bursts, after which other factors often supervene, leading to after-discharge or fatigue.

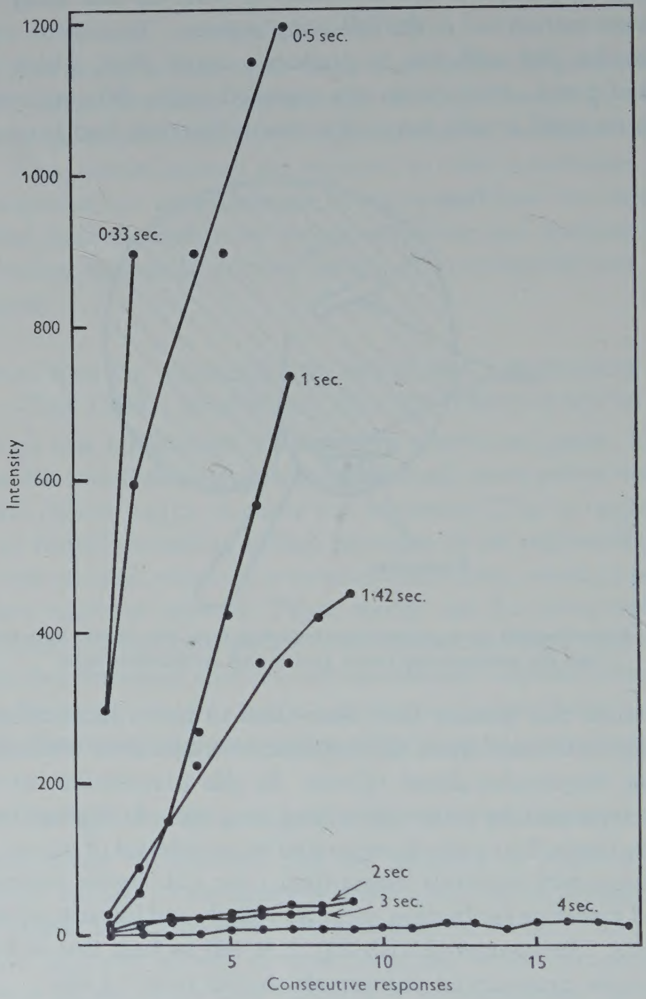


Fig. 2. Increment of intensity of consecutive responses at different frequencies of stimulation. The first response appeared on the 3rd shock, and abscissae represent successive responses. Intensity in arbitrary units; where summation occurred, the intensity measured was the increment over the previous response. Figures on each curve show interval between stimuli in each burst. Recording from entire animal. Temp. 15·7° C.

Facilitation induced by a burst of stimuli persists for several minutes in some specimens (at least 10 min., the longest interval studied). It appears from these results that repetitive stimulation results in progressive augmentation of facilitation, which takes a long time for complete decay: small residual levels may persist for several minutes, thus influencing subsequent responses. A similar persistence of

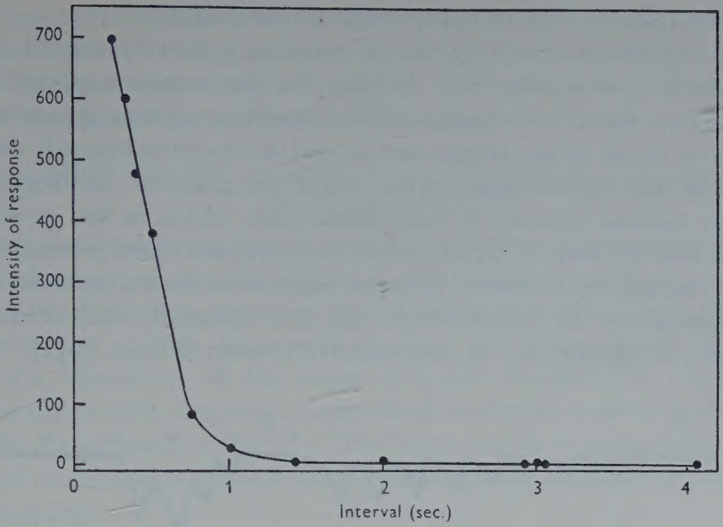


Fig. 3. Facilitation-duration curve. The specimen was stimulated with pairs of shocks, the pulses in each pair separated by intervals ranging from 0·2 to 9 sec. Ordinates represent the intensity of the flash (a single flash to 2 shocks). Abscissae show interval between stimuli. Temp. 18° C.

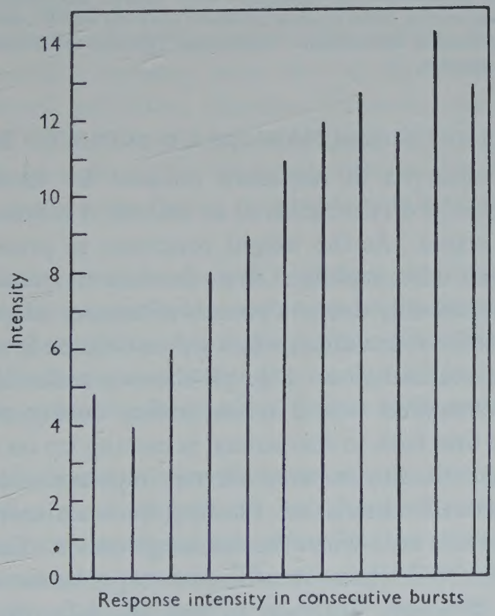


Fig. 4. Facilitation shown in successive periods of stimulation. The specimen was stimulated with bursts of 10 shocks at 1/sec., each burst separated from the preceding one by an interval of 1 min. Each vertical line represents the intensity of the flash evoked by the 10th stimulus in each burst. Temp. 17° C.

small residual levels of facilitation has also been observed in muscular responses of sea anemones (Ross & Pantin, 1940; Pantin & Dias, 1952).

In these experiments, making use of repeated bursts of stimuli, facilitation manifests itself in yet another way. Initially, the first response appears on the 3rd stimulus; in later bursts with continued stimulation, a response appears on the 2nd or even the 1st shock. When fatigue sets in, and the response intensity declines, the first overt response appears again on the 2nd or 3rd stimulus. In these instances it appears that residual amounts of facilitation, when added to that produced by 1 or 2 shocks, raise the level of facilitation to threshold and cause premature flashing. In a similar manner, the facilitated retractor response of the sea anemone *Bunodactis* regularly appears on the 2nd stimulus, but in a repeatedly excited animal small responses to a single stimulus are encountered (Pantin & Dias, 1952).

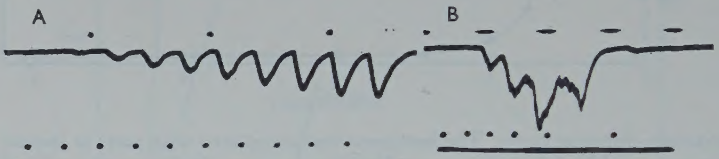


Fig. 5. Comparison of electrical and mechanical stimulation. A: response to a burst of 10 shocks at 1/sec. Dots below represent nervous impulses. The 1st flash appeared on the 2nd pulse. Time scale 14/min. B: responses to mechanical stimulus (dropping a weight on the rachis). Straight line below: duration of mechanical stimulus. Dots below: successive nervous impulses. In addition to propagated waves, there is some localized glowing which contributes to the deflexions in this record of mechanical stimulation. Time scale 72/min. All recordings made through an aperture 2 cm. in diameter.

FACILITATION IN MECHANICALLY INDUCED RESPONSES

Facilitation is also observed in responses induced by tactile stimulation. By dropping a weighted point on the rachis of an animal, it is possible to evoke one or several luminescent waves. As the weight continues to press on the animal the waves, succeeding each other rapidly at first, decrease in frequency and amplitude and die away. This is probably due to a process of sensory adaptation, which is also visible on repeated tactile stimulation, when waves become fewer, less intense, and finally give way to a localized glow. Fig. 5B shows a series of flashes induced by tactile stimulation, compared with a series arising during electrical stimulation (Fig. 5A). Since the first flash in this animal is coming up on the second impulse, it is possible to estimate the succession of nervous impulses underlying the luminescent discharge during tactile stimulation. Flashing shows an interval of about 0·25 sec. at first; this increases to 1 sec., when the discharge ceases. Facilitation of the first three responses is observed when intensities of response are measured, viz. 5, 8, 10·5, 6, and 1 mm. deflexion. Intensity of response falls off as interval between impulses lengthens.

Under tactile stimulation the intensity of successive flashes is governed initially by facilitation, but sensory adaptation soon intervenes, rendering the animal insensitive to further stimulation. The degree of response to tactile stimulation shows much variation among different animals. Nevertheless, we may conclude that a propagated luminescent wave is the result of a battery of impulses evoked by sensory stimulation. The luminescent response of *Renilla*, in this respect, closely resembles sphincter contraction of *Calliactis*, in which tactile stimulation releases a series of impulses and produces a facilitated response (Pantin, 1935*a*).

LATENT PERIOD, DURATION OF RESPONSE, AND RATE OF CONDUCTION

Latent period and rate of conduction were determined simultaneously by a recording method making use of two slits (Fig. 1). One slit lay immediately under and in advance of the electrodes; the other several centimetres away. Each slit was about 3 mm. wide and 15 mm. long, with the long axis perpendicular to the line of advance of a wave-front. A few shocks at a slow frequency (*c.* 1/sec.) were administered and records obtained. Under these conditions latent periods of 0·12 sec. are usually encountered (17–19° C.). The latent period so measured is the sum of latencies of transmission systems (nerve fibres and neuro-photocyte junctions), and of the photocytes.

Examination of the records shows that the latent period of the response is affected to some extent by the frequency of stimulation. In the majority of specimens, the latent periods are fairly constant at frequencies of 1/sec. or less. Moreover, the latencies of consecutive responses usually remain constant during a burst of stimuli at any one frequency below 2/sec. At temperatures of 17–20° C., the mean latency is 0·12 sec. At a higher frequency, 3/sec., it is found that the latent period of successive responses usually increases some two- or threefold during a burst of 10 shocks. There is much individual variation, however, and in some animals the increase in latent period may be as much as tenfold in a series of 10 shocks at higher frequencies. This is brought out in the measurements, shown in Table 1, of latent periods of successive responses from a specimen stimulated at different frequencies (cf. Fig. 6A, B).

The increase in latency which occurs at high frequencies could be taking place at one of three loci: at the synapses of the nerve net, at the neuro-photocyte junctions, or in the photocytes themselves. A change at either of the first two loci seems more probable, but specific data are not at hand to resolve this point. It is suggested that prolongation of latency may be the result of fatigue of the transmitter mechanism at high frequencies.

Conduction speed measured over distances of 35–41 mm. varied from 6·66 to 10·15 cm./sec., with a mean of 8·77 cm./sec. (15·9–17·2° C.). There is a certain amount of variation from one animal to another, but the rates obtained from any one animal tend to be reasonably constant. The values obtained are of the same order as those found by other workers for *Renilla*, viz. 6·5 cm./sec. at 15° C., and 7·39 cm./sec. at 21° C. (Parker, 1920), and 4–5 cm./sec. at 21° C. (Buck, 1955). They conform to slow transmission speeds found in other coelenterates, viz. 12–14 cm./sec. for transmission of muscular excitation in *Metridium* (21° C.), and 4–120 cm./sec. in different pathways of *Calliactis* (18–20° C.) (Parker, 1920; Pantin, 1935*b*).

Temporal characteristics of the luminescent response at one locus were investigated by slit-recording (Fig. 6A, B). Measurements of response parameters from oscilloscope records have provided the following data. At a frequency of 1/sec., the duration of the weak 1st response is 0·25 sec. With continued stimulation the response increases in amplitude and apparent duration. After a series of 10 shocks the response, having reached plateau level, has a duration of 1 sec. The response-curve rises rapidly to a peak and decays more slowly. Maximal height is reached in 0·22 sec. (8th response, duration 0·99 sec.). Total decay time from maximum occupies 0·77 sec.; time for half-decay is 0·15 sec.

Table 1. *Latent period of consecutive responses (sec.)*

Interval (sec.)	Response							
	1	2	3	4	5	6	7	8
2·9	0·12	0·12	0·12	0·12	0·12	0·13	0·14	0·13
1·4	0·14	0·15	0·15	0·16	0·15	0·15	0·15	0·16
1·0	0·19	0·30	0·43	0·54	0·62	0·81	—	—
0·67	0·14	0·30	0·49	0·65	0·91	—	1·11	—
0·50	0·29	0·62	0·91	1·24	—	—	—	—

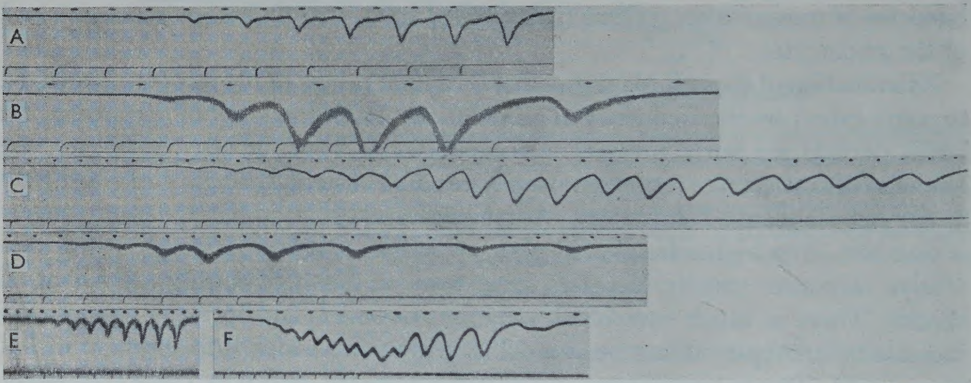


Fig. 6. Luminescent responses in *Renilla* produced by electrical stimulation. A, B: single slit-recordings of an animal stimulated at 42/min. (A), and 90/min. (B). Amplification of A and B, × 10 and × 1, respectively. Temp. 17° C. C, D: influence of successive bursts of shocks on after-discharge. C is the 7th burst in the series; previous bursts had failed to elicit after-discharge. In C there is long after-discharge, lasting 18 sec. Fatigue appears in D, in which response-intensity is much reduced, and there is little after-discharge. Amplification, C, × 1; D, × 10. Temp. 15·4° C. E, F: induction of after-discharge by raising the frequency of stimulation. Repeated stimulation at 1/sec. (bursts of 10 shocks) failed to induce after-discharge (E); whereas a burst of 10 shocks at 3/sec. induced after-discharge lasting 4 sec. (F). Temp. 18° C. Time scale, all records, 72/min.

The apparent lengthening of response with increase in amplitude may, in part, be an artificial effect determined by sensitivity of the recording apparatus. That is to say, the weak initial flashes are not reaching threshold sensitivity of the recording apparatus until some time after they have actually commenced. Buck (1955), however, points out that the autozooid calices produce a less intense but longer lasting glow than the siphonozooids. Moreover, the glowing of the autozooids appears, from visual observation, to be brought up by continued stimulation. There, is then, the additional possibility that apparent lengthening of the response with continued stimulation may be real, and result from differences in the excitability and activity of different luminous centres.

OBSERVATIONS ON HIGH-FREQUENCY STIMULATION

REFRACTORY PERIOD

Slit recordings of animals stimulated at high frequencies bring out certain additional features not evident in recordings from the whole animal (Nicol, 1955). Beginning with a frequency of 1/sec., and becoming more pronounced at higher frequencies, there is some summation of consecutive responses, i.e. each flash fails to decrease to zero before the next one begins (Fig. 6B). The increase in response-intensity secured by higher frequencies, up to 2–3/sec., results, therefore, from a combination of summation and facilitation at each locus of response (photo-emissive area). Despite some basal level of summation, the separate responses remain distinct and are imposed on a constantly glowing background.

At higher frequencies the intensity of response falls off (Fig. 7). At a frequency of 2/sec., initially at least, there is 1 flash for each stimulus beginning with the 3rd shock. With continued stimulation the exact correspondence ceases, and the animal gives only 6–7 flashes for 10 shocks, i.e. towards the end of each burst the animal is no longer responding to each stimulus. With higher frequencies this effect becomes more pronounced, and the animal responds only to every 2nd or 3rd stimulus. The refractory period lies around 0·2–0·5 sec., the exact value varying with the condition of the specimen. Consequently, the fall-off in intensity which is observed when specimens are stimulated with bursts containing the same number of shocks, but at increasing frequencies, is due in part to reduction in number of responses (Fig. 7). Thus, with a stimulation interval of 0·5 sec., there are 8 flashes to 10 shocks; with an interval of 0·3 sec., 5 flashes; and with an interval of 0·18 sec., 3 flashes. Now, facilitation is manifested in consecutive flashes, and depends on number of preceding impulses, and impulse interval. The increase in refractory period which occurs at higher frequencies results in fewer stimuli being effective, and these at longer intervals. The over-all result will be a decrease in facilitation and intensity of response.

These observations show that upper levels of facilitation are limited by the refractory period. Whether the refractory period thus made evident belongs to the excitatory system (nerve net and neuro-effector junctions) or to the photocytes is not resolved by the character of the experiment. By stimulating from two loci, Pantin (1935*a*) has defined quantitatively the refractory period of the nerve net of the sea anemone *Calliactis*. In this animal the relative refractory period lasts 0·5 sec., while the absolute refractory period is 0·05 sec. This value for absolute refractory period appears to be far shorter than the limiting intervals discovered in *Renilla*, but the relative refractory periods in the two animals appear to be similar. In so far as data from one animal can be used to interpret the responses of another, it may be supposed that the relative refractory period of 0·5 sec. in *Renilla* is a property of the nerve net.

The duration of the refractory period is affected by fatigue, just as are certain other features of excitation. It has been observed that continued stimulation at high frequencies lengthens the refractory period. In one specimen, for example, which was stimulated with bursts of 15 shocks at 3/sec., the animal responded with 1 flash per shock for stimuli 2 to 8, and thereafter only to alternate shocks. On repeating with another burst at the same frequency, the animal now responded only to alternate shocks. In *Calliactis* Pantin (1935*a*) notes that the threshold rises when batteries of more than two stimuli are administered, the intervals between the stimuli lying within the relative refractory period. It is suggested that this may be owing to lengthening of the absolute refractory period, but the effect may be complicated by local sensory fatigue.

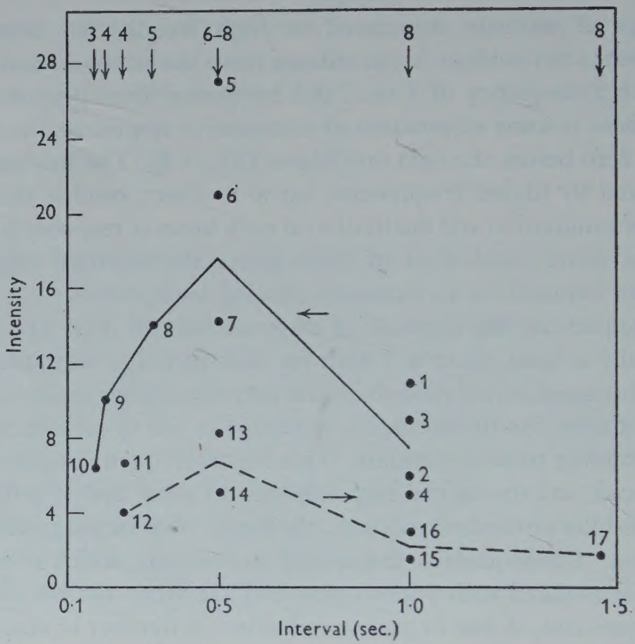


Fig. 7. Influence of frequency of stimulation on intensity of response. Each point on the curves represents the intensity of response to the last shock in a burst of 10 pulses; the numbers beside the points indicate sequence of stimulation. Solid line, a run with increasing frequencies; broken line, a return run with decreasing frequencies. Arrows and figures above refer to number of responses produced by a burst of 10 shocks at different frequencies. Since the 1st response appeared on the 3rd stimulus, the maximal possible number of responses with 10 shocks, excluding after-discharge, was 8. Recording from a narrow slit. Temp. 15–17° C.

Previous work has shown that strong electrical stimuli, several times threshold strength, produce very bright and prolonged responses, which come up on the first shock (Nicol, 1955). Several characteristics of the response are worth examining at this point. The bright response produced by a strong shock is usually a uniform glow covering the entire rachis, and it may last as long as $\frac{1}{2}$ min., in contrast to the short flashes evoked by threshold stimuli. It is sometimes followed by long trains of successive waves, quickly decreasing in frequency and amplitude.

The precise characteristics of responses to strong shocks vary among different animals, but two facts suggest that the response itself is produced by repetitive discharge in the nerve net, viz. an occasional preliminary small wave, and the frequent train of successive waves. To secure further information bearing on this hypothesis, a study has been made of the latent period of responses induced by strong shocks. This turns out to be 0·3–0·4 sec., i.e. 2 or 3 times normal latency. The long latency, thus revealed, excludes direct stimulation of the photocytes as an operative factor. Since normal latency is only some 0·12 sec., there is a further interval of some 0·15–0·25 sec. during which one or two impulses could succeed the first to produce a strong facilitated response.

If repetitive discharge is occurring, initially at least, it must be taking place at such a rate that fusion of consecutive responses takes place. It may be that the high level of facilitation built up by the rapid discharge of a few impulses is sufficient to maintain a sustained glow in the photocytes. Certainly, the bright even glow produced by strong electrical stimulation is quite different from the trains of waves induced by threshold stimulation. It closely resembles the bright glow produced by strong tactile stimuli, and both phenomena may have a similar neural causation.

AFTER-DISCHARGE AND MAINTAINED RHYTHMIC FLASHING

Under certain conditions some animals continue flashing for some time after stimulation has ceased. This after-discharge is evoked by tactile and electrical stimulation. Specimens, however, vary greatly in excitability. In some animals a single tactile stimulus or a few electrical shocks evoke a post-stimulatory series of waves; in others repetitive or prolonged stimulation is required. After-discharge varies greatly in duration, from one or a few waves to prolonged flashing lasting many minutes. Initially, at least, the waves in a post-stimulatory discharge arise from the area of stimulation, and run regularly over the surface of the rachis. When a specimen is highly excited, by prolonged electrical or tactile stimulation, the whole surface of the rachis flashes continuously in a shifting and confusing pattern.

After-discharge is obviously a condition dependent on excitability of the nerve net, and it is possible to explore its characteristics by controlled stimulation. The following conclusions are based upon the examination of many animals.

When a short burst fails to elicit post-stimulatory discharge, a long burst of stimuli frequently will do so. Again, when a single short burst is ineffective, a series of similar bursts will often produce post-stimulatory flashing. The effective interval between bursts for production of after-discharge is at least 7 min. (Fig. 6C, D).

The number of flashes in an after-discharge depends on the number of stimuli. Whereas, with a short burst, only one or a few post-stimulatory flashes may appear, a long burst may give rise to a prolonged period of post-stimulatory flashing. Similarly, with repeated bursts there is an increase in the number of post-stimulatory flashes, following each burst of shocks. Thus, in one animal stimulated by bursts of shocks (5 pulses at 1/sec.), with 5 min. between bursts, the first burst produced no after-discharge, the second produced 8, and the third 14 flashes after stimulation had ceased.

After-discharge is readily induced by high-frequency stimulation. Thus, it has been frequently observed that when a burst of shocks at a low frequency (1/sec. or less) is ineffective, a burst of an equal number of shocks at a higher frequency (3/sec.) results in much post-stimulatory flashing (Fig. 6E, F).

In post-stimulatory discharge induced by tactile and electrical stimulation, the frequency of flashing is usually rapid at first, and shows a tendency to decline. Initial periodicity is of the order of 0·5–1 sec. and increases to 2–3 sec. after 15–30 flashes (Figs. 6C, F, 8). These are approximate values to give some idea of the magnitudes involved. As the interval between consecutive flashes increases during after-discharge, there is a corresponding decline in the intensity of consecutive flashes (Fig. 6C). This results in large part from decay of facilitation as the interval between impulses lengthens.

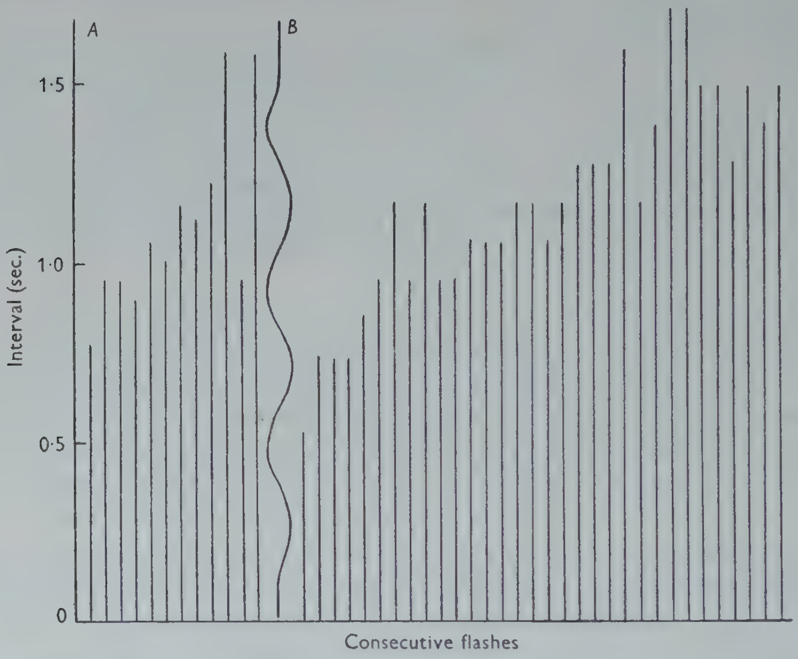


Fig. 8. General decline in frequency of pulses during a period of post-stimulatory discharge following electrical stimulation (*A*), and tactile stimulation (*B*). Ordinates, interval (sec.) between consecutive flashes. Temp. 18·1° C.

Repetitive flashing, like facilitation, is subject to fatigue. After a period of prolonged after-discharge, induced by a burst of electrical shocks, similar stimulation may be ineffective in producing after-discharge, or may elicit only one or a few post-stimulatory flashes (Fig. 6D). Nevertheless, by increasing the number of stimuli, or frequency of stimulation, post-stimulatory flashing may be revived or prolonged in such fatigued animals.

THE EFFECTS OF CERTAIN DRUGS ON THE LUMINESCENT RESPONSE

Several drugs were tested on *Renilla* in order to determine whether they would evoke luminescence or affect the facilitatory process. These drugs were adrenaline, acetylcholine, eserine, curare, and nicotine. With the exception of nicotine the tests were negative, and gave no information about transmitter action in this animal. Nicotine in high concentration (1/1000) greatly reduced the response and caused irregular and weak flashing. It appears to have a twofold effect, excitatory on the nerve net, and depressive on the photocytes.

DISCUSSION

This paper has been concerned with three aspects of the luminescent response of *Renilla*, namely a finer resolution of response parameters, facilitation, and the prolonged excitatory state manifested in after-discharge. Various lines of evidence indicate that each electrical stimulus gives rise to a nervous impulse which traverses the entire nerve net of the rachis. The progress of the luminescent wave across the rachis is an index of transmission of excitation in the nerve net, and measurements of conduction velocities obtained by this means give a value of about 9 cm./sec. (15·5° C.).

Earlier work has shown that the nerve net of *Renilla* is unpolarized and that transmission occurs readily in all directions (Parker, 1919; Buck, 1955). There is indirect evidence that the nerve net consists of a meshwork of short distance neurones (Nicol, 1955). Although histological studies on *Renilla* are wanting, it seems likely from our knowledge of neurones in other coelenterates (medusae, sea anemones) that these nerve cells make contact with one another by synaptic junctions (discussion by Pantin, 1950, 1952). Normally each impulse successfully bridges these synapses and invades the whole nerve net, but under certain experimental conditions decremental conduction can be induced, whereby several impulses must intervene in order to force a crossing from one region to another, owing to intervention of synaptic resistance (Nicol, 1955). It has been concluded that facilitation of the luminescent response, observed in these studies of *Renilla*, occurs terminally at the level of the effectors (Buck, 1955; Nicol, 1955).

Facilitation of the luminescent response of *Renilla* shows essentially the same characteristics as those observed in the quick muscular responses of actiniarians and Scyphomedusae (Pantin, 1935*a*; Bullock, 1943). The question arises, where is the facilitatory process taking place? Pantin (1935*a*, *b*) has argued that facilitation of the marginal sphincter in *Calliactis* is a neuro-muscular effect such that each stimulus in a series reaches more and more individual muscle fibres (recruitment of effector cells). He has found that a single stimulus applied directly to the muscle causes it to contract, whereas several impulses are required to evoke contraction when the muscle is stimulated via the nerve net. From this it is concluded that the muscle itself is capable of responding to the first excitatory impulse that reaches it. Since each impulse invades all regions of the nerve net, it follows that the facilitatory process occurs at the neuro-muscular junction (Pantin, 1935*c*).

Now, the same argument can be fairly applied to the luminescent responses of *Renilla*. First of all we may note that under repetitive stimulation a tiny spot of light appears directly beneath the electrodes on the first stimulus, although several shocks are usually required to give rise to a propagated luminous wave. The photocytes, therefore, are capable of responding directly to a single excitation. Now, conduction of each impulse is widespread, involving the whole nerve net, although two or three impulses must intervene before a luminescent wave appears. It follows, therefore, that the facilitatory process is taking place at the neuro-effector junction, between the nerve fibres and the photocytes. This conclusion would appear to dispose of the possibility that facilitation results from triggering and augmentation of some chain of reactions leading to light emission within the photocytes.

We find that several impulses are required to bridge the gap between nerve fibres and photocytes, thus exciting the latter. The subsequent increase in height of successive responses depends on recruitment of additional photocytes as facilitation builds up. This thesis presumes that there is variation in the threshold of different photocytes, or possibly variation in activity levels of the transmitting mechanism at different nerve fibre terminals. Evidence for variation in the threshold of different photocytes is forthcoming from visual observations. When the eye is dark-adapted, and *Renilla* is given a series of pulses at a low frequency, it is observed that the first few stimuli, although generally ineffective, sometimes cause a few isolated points of light to appear at widely separated places *all over* the rachis. A related effect is observed in animals stimulated at very low frequencies (interval >3 sec.). Under these conditions not every stimulus is effective, and there may be gaps of 3, 4 or more shocks before the next flash or transmitted wave appears. But during these dark periods it frequently happens that odd scattered points of light develop on the rachis in response to a stimulus. It seems that these few zooids which are responding are below the threshold of the majority, and similarly, within each zooid, there is possibly variation in the threshold of individual photocytes.

Some previous workers, who have studied muscular responses of actiniarians, have discussed the nature of neuro-muscular transmission in this group (Ross & Pantin, 1940; Ross, 1945). From various lines of evidence they advance the hypothesis that neuro-muscular transmission in anemones involves two distinct processes, viz. a process of excitation; and a process of sensitization of the neuro-muscular junction whereby excitation of the muscle by the nervous impulse can become effective. Among the evidence cited are the following facts. (1) A single stimulus produces no response, and the response takes place on the second stimulus if it occurs at all. (2) When the interval between stimuli is lengthened, the response to the third stimulus in a series disappears at the same time as the response to the second, and it is not possible to obtain two apparently ineffective stimuli before the first response. The observations just cited have been interpreted as evidence against a transmitter hypothesis. They have been selected for consideration because comparable data are available from *Renilla*.

Evidence has been presented for *Renilla* which shows that a luminescent response usually occurs on the 2nd or 3rd shock; subsequent responses increase progressively in intensity; intensity of response depends on interval between stimuli, i.e. facilitation builds up and decays in a predictable manner. These observations are compatible both with a theory of a chemical transmitter, and with a theory of sensitizer involved in excitation. There is one condition, however, in which luminescence appears on the first stimulus, viz. shortly after a previous period of stimulation. It thus appears that a sufficient level of facilitation can persist from a previous period of stimulation to make a single shock effective. Moreover, we have seen that when the stimulation-interval is progressively lengthened, the response, instead of coming up on the 2nd or 3rd shock, now appears after an increasingly greater number of shocks. Together, these various lines of evidence strongly suggest that neuro-photocyte transmission involves release and decay of a facilitatory agent. The evidence at hand, for *Renilla*, does not demand the postulation of a sensitizer separate from the excitatory agency. Under normal conditions it appears that several impulses must intervene before facilitation reaches a level sufficient to excite the photocytes; but when some residual facilitation is already present, a single impulse may release enough facilitatory agent which, when added to that already present, will excite a response. In this connexion it is noteworthy that residual traces of facilitation may persist for at least 10 min. Moreover, when the interval is lengthened an increasingly greater number of impulses must intervene before sufficient facilitatory agent accumulates to excite a response.

Whereas the increment in height of successive responses depends on facilitation at the neuro-effector junction, repetitive discharge is a property of the nerve net. It is manifested in interpolated waves which appear during a burst of electrical stimuli. And it occasions the prolonged bouts of post-stimulatory flashing seen in some specimens following electrical or mechanical stimulation.

Repetitive discharge apparently originates in the neurones of the nerve net. At low levels of excitation, one or a few extra waves appear at the site of stimulation. There are instances, however, where rhythmic flashing may continue for some time from the site of stimulation after the stimuli have ceased. Evidence for after-discharge, of course, is indirect, and is derived from observation of the character and course of post-stimulatory flashing. It appears as if certain neurones in the nerve net, when sufficiently excited, continue to discharge rhythmically after stimulation has ceased. They can be primed, so to speak, to higher levels of rhythmic activity by increasing the frequency or duration of stimulation. With time, their excitability declines, and rhythmic discharge, as manifested in flashing, fades away.

Studies now available provide information for regulation of three effector systems of coelenterates, viz. muscular systems, luminescent systems and cnidae. Of these, the muscular and luminescent systems are under nervous control, whereas cnidae are independent effectors. Contractile tissues are universally present among coelenterates; luminescent tissue, on the other hand, occurs sporadically and probably has been acquired independently on many occasions in this group. There are certain striking similarities in neuro-effector regulation between the luminescent responses of sea pens and the muscular responses of medusae and anemones. There is some reason for believing, therefore, that the mode of regulation of the luminescent response has been determined by the functional organization of a nervous system already concerned with phasic and tonic muscular activity. To this nervous system has been added regulation of the luminescent response, apparently with little change in functional characteristics.

SUMMARY

1. Luminescence has been studied in the sea pansy *Renilla köllikeri*, by means of photo-electric recording, and the mode of nervous regulation investigated.

2. The luminescent response is under control of a non-polarized nerve net and is subject to facilitation which occurs terminally, at neuro-photocyte junctions.

3. Facilitation is analysed in detail, and a facilitation-decay curve presented (Fig. 3). Between successive bursts of stimuli, facilitation may persist for some 10 min.

4. Certain response parameters were measured. Latent period is 0·12 sec.; the response lasts 1 sec., and maximal intensity is reached in 0·22 sec. Conduction speed is 9 cm./sec. at 16–17° C.

5. The refractory period of the response, as determined by high frequency stimulation, is 0·2 sec. It is affected by fatigue and increases under repetitive stimulation.

6. Under repetitive and prolonged stimulation, the animal passes into a hyper-excitatory state and luminous waves continue to arise long after stimulation has ceased. Conditions affecting this post-stimulatory discharge are examined.

7. Comparison of facilitatory processes in quick muscular responses of sea anemones and luminescent responses of sea pens shows that they are essentially similar. From visual observations it appears that facilitation operates by recruitment of photocytes. Luminescence is of sporadic occurrence among coelenterates, and has been independently evolved on many occasions. It is suggested that its mode of regulation has been determined by the characteristics of the nerve net primarily concerned with control of muscles.

This work was carried out at the Scripps Institution of Oceanography, La Jolla, California, during tenure of a Guggenheim Fellowship. I should like to repeat the acknowledgements made at the end of my previous paper (1955) dealing with *Renilla*.

REFERENCES

Buck, J. (1955). Bioluminescence in *Renilla*, in relation to nerve net physiology. Personal communication.

Buck, J. & Coyle, T. D. (1955). Interrelations between stimulus and luminous response in *Renilla*. Personal communication.

Bullock, T. H. (1943). Neuromuscular facilitation in Scyphomedusae. *J. Cell. Comp. Physiol.* **22**, 251–72.

Nicol, J. A. C. (1955). Observations on luminescence in *Renilla*. *J. Exp. Biol.* (in the Press).

Pantin, C. F. A. (1935*a*). The nerve net of the Actinozoa. I. Facilitation. *J. Exp. Biol.* **12**, 119–38.

Pantin, C. F. A. (1935*b*). The nerve net of the Actinozoa. II. Plan of the nerve net. *J. Exp. Biol.* **12**, 139–55.

Pantin, C. F. A. (1935*c*). The nerve net of the Actinozoa. IV. Facilitation and the 'staircase'. *J. Exp. Biol.* **12**, 389–96.

Pantin, C. F. A. (1950). Behaviour patterns in lower invertebrates. *Symp. Soc. Exp. Biol.* **4**, 175–95.

PANTIN, C. F. A. (1952). The elementary nervous system. *Proc. Roy. Soc.* B, **140**, 147–68.

PANTIN, C. F. A. & DIAS, M. V. (1952). Excitation phenomena in an actinian (*Bunodactis* sp.?) from Guanabara Bay. *An. Acad. bras. Cienc.* **24**, 335–49.

PARKER, G. H. (1919). The organization of *Renilla*. *J. Exp. Zool.* **27**, 499–507.

PARKER, G. H. (1920). Activities of colonial animals. II. Neuromuscular movements and phosphorescence in *Renilla*. *J. Exp. Zool.* **31**, 475–515.

ROSS, D. M. (1945). Facilitation in sea anemones. I. The action of drugs. *J. Exp. Biol.* **22**, 21–31.

ROSS, D. M. & PANTIN, C. F. A. (1940). Factors influencing facilitation in Actinozoa. The action of certain ions. *J. Exp. Biol.* **17**, 61–73.

THE NERVES AND MUSCLES OF MEDUSAE

III. A DECREASE IN THE REFRACTORY PERIOD FOLLOWING REPEATED STIMULATION OF THE MUSCLE OF *RHIZOSTOMA PULMO*

By G. A. HORRIDGE

Department of Zoology, University of Cambridge

(*Received* 2 *February* 1955)

INTRODUCTION

In his paper on *Aurellia* and other medusae, Bullock (1943) introduces a method which makes possible the measurement of the refractory periods of the nerve and muscle in a strip cut from the bell of a medusa. During a repetition of this experiment, using the Mediterranean form *Rhizostoma pulmo*, it was soon noticed that the responses depended on the previous activity of the preparation, and this could not be explained by the known characteristics of coelenterate muscle. Bullock's method of stimulating a strip of the bell can be represented diagrammatically as in Fig. 1, where time is plotted downwards. A single stimulus at *A* is followed by a wave of contraction down the strip to *B*. If a second stimulus is applied at *B* after such an interval that it falls within the refractory period of the nerve at *B*, there will be no further response. However, if the stimulus falls when the nerve can again respond, but when the muscle is still refractory, a second wave of excitation will be propagated back along the strip. Although the muscle is refractory at *B* this second wave will bring about a second contraction at *A* if the strip is sufficiently long for the muscle at *A* to have emerged from its refractory period. Ideally then, two contractions are observed at the end *A* and one contraction at the end *B*. These contractions are represented at the sides of the diagram. The interval between the two responses at *A* depends on the length of the strip, the interval between the stimulus at *A* and that at *B*, and the velocity of propagation in each direction.

METHODS

Half of a bell of a large specimen of *Rhizostoma* was taken, and the regions round the mouth and along the margin were removed. This produced a curved strip about 25 cm. long and 5 cm. wide, which was pinned down in sea water in a way that allowed the contraction at each end to be recorded separately. In the absence of the tentaculocysts there were no spontaneous movements. The responses to stimulation were recorded on a smoked drum with light isotonic levers. Stimulation was by single break shocks from two induction coils, one for each end of the strip. The method of stimulating the two ends separately with a constant interval between stimuli was as follows. A heavy pendulum, of adjustable period, was fitted with a light contact which worked on a hinge, *H*, Fig. 2. Below the pendulum was clamped

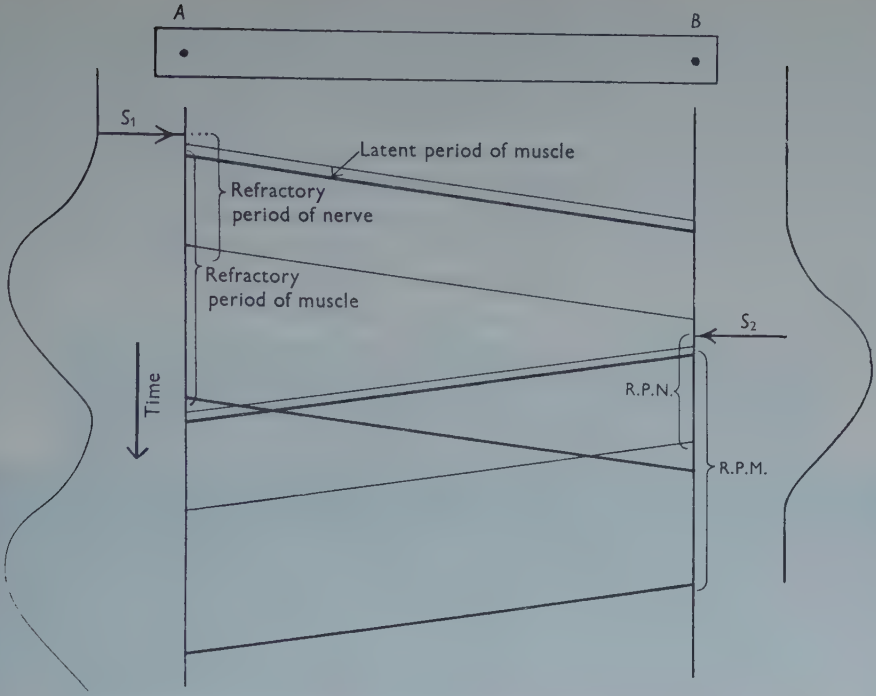


Fig. 1. A representation of the propagation of excitation following the first stimulus S_1 and the second S_2 with an indication of the response of the muscle. The time scale is not known accurately and is therefore not shown.

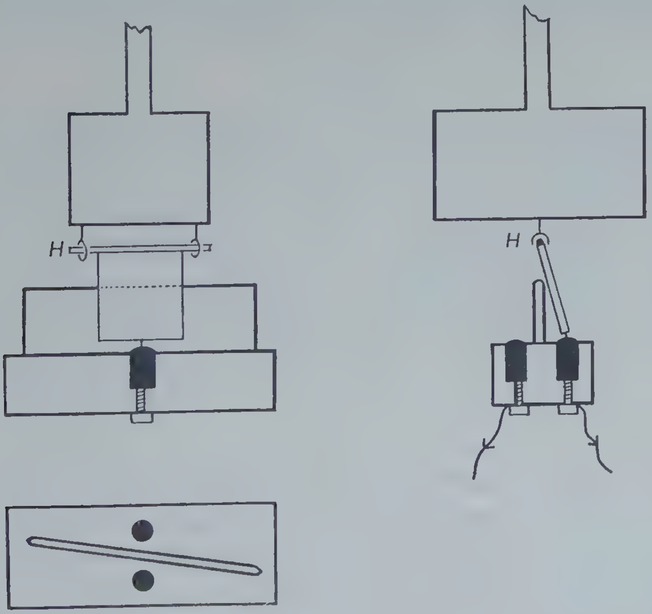


Fig. 2. The arrangement of the contact on the pendulum to give a constant interval between stimuli which are destined for opposite ends of the strip of tissue.

a block of insulating material in which two cups full of mercury were arranged as shown (black in the figure). Between these cups a partition was fixed in such a way that during the swing of the pendulum in one direction the contact would pass through the mercury in one of the cups, and during the swing back it would pass through the other. With the pendulum at rest the contact must be exactly between the mercury cups to ensure a constant interval between the two contacts irrespective of their order. The pendulum was always released from a fixed point to swing with the same amplitude on each occasion and was caught after each full swing. Each mercury cup was connected in the primary circuit of an induction coil, the secondary being adjusted so that only the 'break' shock exceeded threshold. To reverse the order of stimulation the pendulum was moved across to begin its swing from the other side. This movement caused a single stimulus, which is seen on the record Fig. 3 marked by a small arrow, α, β, γ.

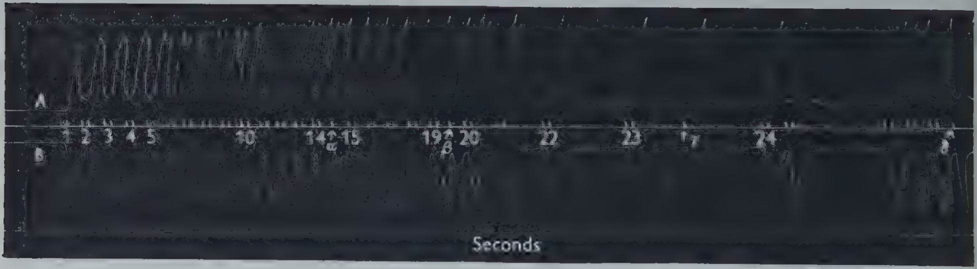


Fig. 3. Records from the two ends *A* and *B* of a strip 25 cm. long from the bell of *Rhizostoma*. Contraction is registered upwards at *A* and downwards at *B*. Between the *A* and *B* traces is the record of stimuli, two break shocks at a constant interval of 1 sec. Over the part of the record from the beginning to the point α and again between β and γ the first stimulus was applied at *A* and the second at *B*; between α and β and between γ and δ the order was reversed.

RESULTS

As can be seen from Fig. 3, the first pair of stimuli produce only a single contraction at each end, but at the end *A* the single contraction soon resolves itself into two parts until at stimulus pair no. 10 the response is characteristic of Bullock's method for measuring refractory periods, with two contractions at *A* and one at *B*. By stimulus pair no. 14 the single response at *B* shows signs of a second contraction. At the point α the order of stimulating the ends is reversed so that *B* receives the first stimulus. The double response now seen at *B* is similar to that formerly at *A*, and the response at *A* shows the small second response formerly at *B*. At β the order is again reversed and the symmetry is again evident. At this point the interval was increased before the next pair of stimuli, and it is seen that the second response at *B* disappears. The second response at *A* (no. 21) is reduced a little, further reduced at no. 22 and even further reduced at no. 23 after the still longer pause. The order of stimulation is again reversed at γ and after a pause of 10 sec. the end *B* receives the first of the pair of stimuli. There is now (no. 24) only a small second response at *B* and no second response at *A*, but a pair of stimuli immediately after give a response similar to those at nos. 15–19.

The progressive appearance of the second response is well shown by the first part of the trace, the decay of the phenomenon is shown by the later responses, and the reversal of ends at α shows that the effect is present at both ends of the strip of tissue. The progressive appearance of the second response at *B* is shown also in Fig. 4, but in this preparation it appears towards the end, rather than at the height, of the first response. In this record the responses at the end *B* for the contractions nos. 1 and 2 show marked facilitation, both in the greater height of the response and in the more rapid relaxation of the second contraction. Bullock called the latter effect 'facilitation of relaxation'. The appearance of the second response in nos. 7–15 (Fig. 4) is accompanied by both of these long-term effects, but this does not mean necessarily that they are causally related to it. It may be objected that the progressive appearance of the second response is due to the facilitation of relaxation, i.e. that the second response has been present from the beginning but cannot show on the record until the muscle has relaxed after the first response. However, an examination of the lower trace of Fig. 3 shows that apart from the first response the rate of relaxation is the same for all; again, in Fig. 4, the second response at the end *A* grows from the end of the relaxation.

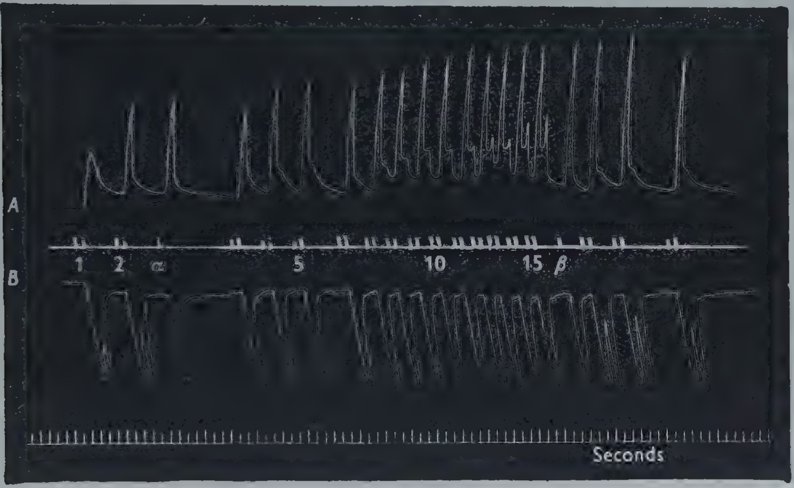


Fig. 4. See legend to Fig. 3. In this preparation the interval between the stimulus pairs was 0·88 sec.

DISCUSSION

Let us first consider the effect at the end *B* (Fig. 3). Here the first contraction of the experiment is a single peak, and after several repetitions a smaller second contraction appears immediately following the first. The first contraction at *B* is due to the first stimulus, which is applied at *A*. Initially there is no second response at *B* because the muscle fibres are all refractory to the second stimulus. The progressive appearance of a second contraction at B may be due to a progressive *increase* in the velocity of the excitation wave from A to B, so that the stimulus at B comes later relative to the excitation arriving from A. Alternatively, it may be due to a progressive shortening of the refractory period of muscle fibres at B. There are no other obvious factors which might undergo a progressive change to give this effect, namely that more and more of the muscle at B is able to give a second contraction.

Now consider the effect at A. At first there is only a single contraction due to the first stimulus, which is applied at A. Progressively there appears a larger and larger second contraction which is due to excitation spreading from the second stimulus which is at B. Initially stimulation at B produces no second contraction at A. This could be interpreted by saying that the stimulus at B comes within the refractory period of the nerve at B, so producing no second excitation. However, a second contraction at A appears progressively, not all at once, as it would do if the effect were due to a shortening of the refractory period of the nerve; for the nerve acts in an all-or-nothing manner and with constant time relations cannot produce a progressively increasing second contraction at A. In fact there must be a second excitation of the nerve at A on every occasion to which the muscle is able to respond with successively greater contractions.

The events at A can be explained by a *decrease* in the velocity from B to A, so that the muscle at A has more time to come out of its refractory period before the nerve impulse arrives from B. But, as shown above, an *increase* in velocity from A to B is required to account for the events at B. When the order of stimulation is reversed the second contraction still persists at both ends. This shows that whatever the effect responsible for the progressive increase of the second contraction, it is maintained irrespective of the direction in which the excitation travels. In fact the velocity of propagation cannot decrease in one direction and increase in the other.

The satisfactory explanation of all the observations is the progressive shortening of the refractory period of more and more muscle fibres. The first stimulus is applied at A, and after an interval a second excitation arrives from B, at first during the refractory period of most of the muscle fibres. Progressively more and more of the muscle fibres have come out of the refractory state when the second excitation arrives, and a larger and larger second contraction appears.

The phrases used in the last paragraph suggest that this sheet of muscle is envisaged as a population of individual fibres, each of which acts in an all-or-nothing manner, in which the strength of the contraction will then depend upon the number of fibres responding to a stimulus. But whether the unit is the individual fibre, part of a fibre or a group of fibres the same explanation would apply. The shortening of the refractory period at the neuromuscular junction has similar time relations to the facilitation of the strength of the response, but it is only a speculation to consider it a part of one general phenomenon.

SUMMARY

Evidence is presented to show the shortening of the refractory period of some of the elements in a population of coelenterate muscle fibres following frequent stimulation of the bell of *Rhizostoma pulmo*.

I would like to thank Dr R. Dohrn and his staff at the Stazione Zoologica, Naples, for the use of their facilities and their kind interest during my visit.

REFERENCE

BULLOCK, T. H. (1943). Facilitation in Medusae. *J. Cell. Comp. Physiol.* **22**, 251.

THE NERVES AND MUSCLES OF MEDUSAE

IV. INHIBITION IN *AEQUOREA FORSKALEA*

By G. A. HORRIDGE
Department of Zoology, University of Cambridge

(*Received* 2 *February* 1955)

INTRODUCTION

The large size of mature specimens of *Aequorea forskalea*, up to 25 cm. in diameter, makes this lepto-medusan a particularly favourable object for physiological work. This paper describes an investigation of the co-ordination of movement in *Aequorea* carried out along lines similar to an earlier study of *Geryonia* (Horridge, 1955).

MATERIAL AND METHODS

The anatomy of *Aequorea* is well known (Hertwig & Hertwig, 1878; Mayer, 1910) and its histology has been studied (Krasinska, 1914). The bell of the adult is saucer-shaped when relaxed and it will be convenient to distinguish three regions:

(*a*) *The manubrium and stomach wall.* In the centre of the bell hangs the manubrium, which is a thin transparent sheet of radial muscle fibres. When this is relaxed the mouth hangs down several centimetres and opens internally to the stomach, which is flat and disk-shaped, with a diameter about half that of the whole bell.

(*b*) *The radial canals and gonads.* From the stomach about seventy radial canals run to the periphery. Along the line of each canal runs a gonad, a flattened tube shaped like the blade of a sickle, hanging by one edge from the subumbrellar surface, and having a muscle strand running in the free edge.

(*c*) *The margin.* The gonads stop short of the margin, which contains two nerve rings and bears the velum and the tentacles, which are more numerous than the radial canals. These features can be seen in Figs. 1, 2.

Mechanical stimulation was usually applied with a glass rod or a piece of wire and electrical stimulation with a pair of platinum electrodes mounted in an insulating handle. Single short pulses of constant current ('square waves', with a duration of a few msec.) were most satisfactory, and the intensity of the stimulus was kept a little above threshold to minimize artifacts which might arise from stimulus escape. The contractions were recorded by writing levers attached with threads to hooks of bent wire embedded in the jelly. This was arranged so that both radial and circular muscle were recorded by the same lever; in the records (see Figs. 3, 4) the twitch-like contraction of the circular muscle is readily distinguished from the slow sustained contraction of the radial muscle.

THE CIRCULAR MUSCLE AND THE SWIMMING MOVEMENTS

The rhythmical contractions of the circular muscle, by which the animal swims, will be referred to as 'the beat'. As in all medusae the bell contracts symmetrically when beating normally, but in *Aequorea* during periods of comparative quiescence occasional local contractions can be seen, and the symmetrical action is not fully developed until the rhythm has been sustained for several minutes.

The beat is co-ordinated by a rapid through-conducting system which is confined to the margin. By recording from two points some 30 cm. apart, the velocity of conduction is found to be 70–90 cm./sec. at 20° C. Section of the ring nerve abolishes this rapid conduction, and in this respect *Aequorea* differs from *Geryonia*, in which the general conduction over the subumbrellar epithelium is as rapid as that in the nerve. It is likely that this rapid conducting system in *Aequorea* can be identified with the broad nerve fibres (Hertwig & Hertwig, 1878; plate VI, fig. 6, *y*) in the lower ring nerve, which sends axons over the subumbrellar epithelium and the velum.

After section of the ring nerve excitation to the circular muscle is still conducted, though more slowly, through the subumbrellar epithelium. By studying the responses of strips cut from the bell it can be shown that conduction takes place in all directions, but more readily in a radial than a circular direction. In one such preparation, the contraction wave traversed 8 cm. in a radial direction at the first stimulus, whereas several stimuli are usually necessary for the contraction wave to be propagated a similar distance in the circular direction. Variation of the strength of the stimulus above threshold has no effect upon either the amplitude of the contraction or the distance to which the contraction wave is propagated. At each successive stimulus the contraction wave spreads further but in any given region the amplitude of the contraction does not increase. In fact the observations indicate a similarity to the all-or-nothing contraction wave of the Scyphozoa.

When the whole bell is beating normally, excitation spreads from one rhythmical centre in the margin via the through-conducting system to all the circular muscles. Any isolated part of the margin beats at its own rate, so that in the normal beat the pace must be set by the centre with the highest natural frequency. A similar situation has been described in *Aurellia* by Pantin & Vianna Dias (1952).

THE RADIAL MUSCLE AND THE FEEDING RESPONSE

The feeding response is made up of a series of movements brought about by groups of radial muscles and co-ordinated by physiological pathways that run largely radially. Food is caught on the tentacles, of which a few contract together; this region of the margin bends inwards towards the mouth; at the same time the manubrium is displaced towards the stimulated region and the mouth opens to receive the food. The whole process usually takes several minutes.

The inward bending of the margin is due to the contraction of the adjoining radial muscle in the region of the radial canals. This is shown by the direction of small folds which appear at right angles to the radial canals, and by the fact that the

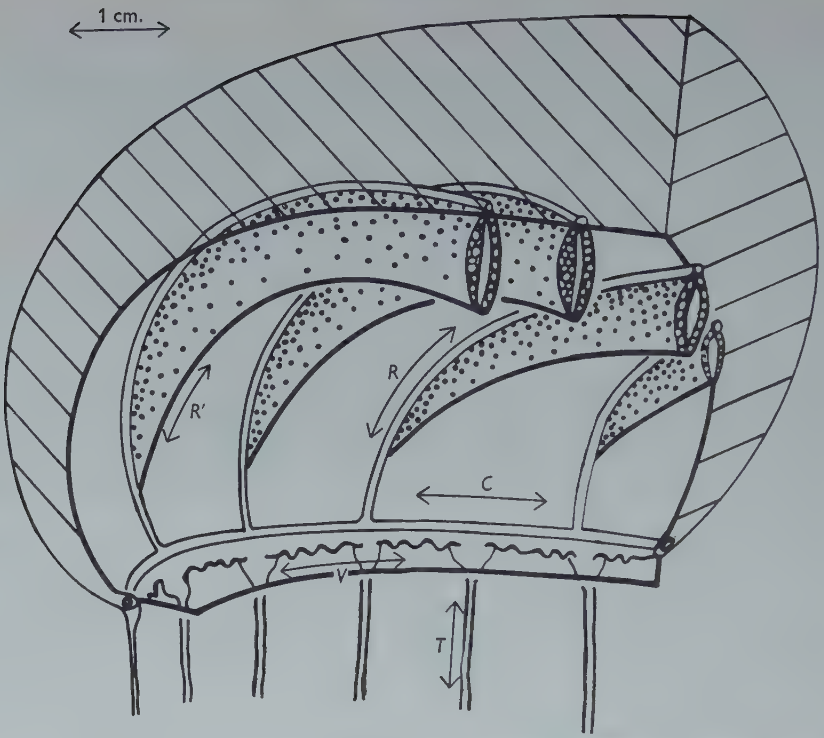


Fig. 1. Part of the bell of *Aequorea* to show the arrangement of the velum, gonads, and muscles. *C*, circular muscle of the bell; *R*, radial muscle of the bell; *R'*, radial muscle of the gonad edge; *T*, muscle of the tentacle; *V*, circular muscle of the velum.

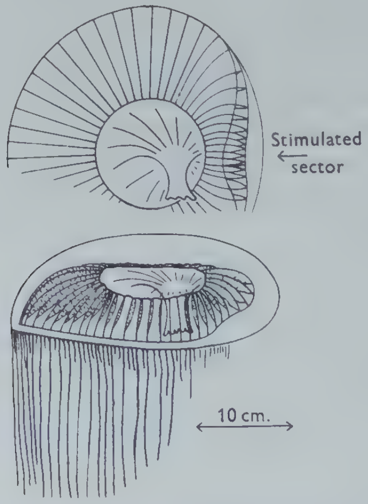


Fig. 2. Following mechanical stimulation of any point on the subumbrellar surface the margin bends towards the mouth, which is displaced laterally to meet it. The response of the radial muscle does not spread all round the bell, though a wave of tentacle retraction may do so if the margin is entire.

radial canals are not drawn closer together as they are during the contraction of the circular muscle. The inward bending is reinforced by contraction of the strand of muscle in the free edge of each gonad. The lateral movement of the mouth is brought about by the radial muscle of the stomach wall.

It can be shown by the familiar method of inter-digitating cuts that in the region of the radial canals excitation to the radial muscle is conducted predominantly in a radial direction, either centrifugally or centripetally. However on the sides of the lamellae of the gonads there is conduction at right angles to the radial line, as shown by the behaviour of the muscle strand along the free edge; if the gonad is divided in several places by transverse cuts, all parts of the muscle strand contract together with the neighbouring radial muscle. In the marginal region excitation to radial muscles spreads in a circular direction across the subumbrellar epithelium. Following stimulation of a tentacle, the radial muscle of the same radius contracts and brings about a local inward movement of the margin. Excitation then spreads circularly for about 10 cm. in both directions, as shown by inward bending of successive regions of the margin, immediately preceded by contraction of the corresponding tentacles (Fig. 2). The velocity of conduction appears to be 0·2–2·0 cm./sec. This circular spread of excitation is not prevented by section of both ring nerves but is prevented by a cut of 1·5 cm. or more into the margin. Co-ordination between tentacles, however, is abolished by a cut in the margin through both ring nerves, as in *Geryonia*.

A spread of excitation in the circular direction is also seen in the wall of the stomach. The lateral movement of the manubrium is due to the contraction of the radial muscles in the same raduis as the stimulus, but after continuous stimulation the excitation spreads round the mouth, which in consequence is widely opened.

The contraction of the radial muscle is slow and sustained, in contrast to the twitch of the circular muscle (Figs. 3, 4). Unlike the latter it is evoked by gentle mechanical stimulation and if electrical stimuli are used, several may be required to produce a response.

In a piece of the radial canal region, deprived of marginal nerves, the radial muscles respond only so long as the stimulus continues to be applied, but with the adjoining ring nerves intact a contraction may sometimes be sustained for several minutes even after a single electrical stimulus.

THE INHIBITION OF CO-ORDINATED SWIMMING DURING THE FEEDING RESPONSE

During the feeding response, or when the radial system is electrically or mechanically stimulated, there are two effects of great interest. First, it can be shown that the excitation in the radial system inhibits the spontaneous rhythm of the beat. Fig. 4 shows a series of spontaneous beats recorded from opposite sides of the bell of an undamaged specimen. Electrical stimulation was applied to the margin, and as the contraction of the radial muscle develops the spontaneous beat comes to an end. Secondly, it can be shown that excitation in the radial system prevents the rapid marginal propagation of the beat, shown above to be distinct from the general

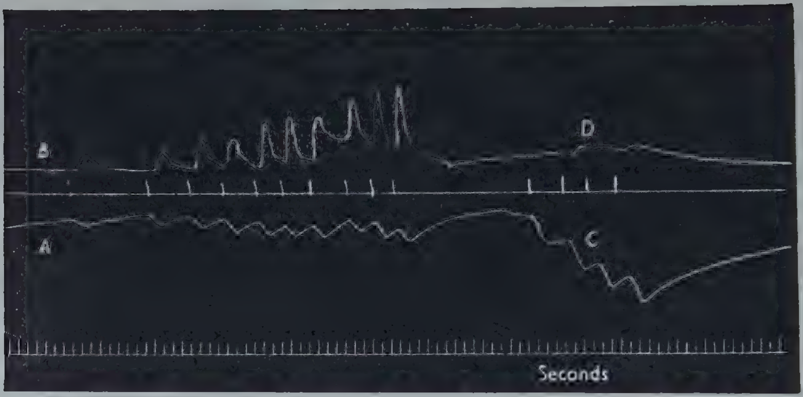


Fig. 3. Simultaneous records from two adjacent parts, *A* and *B*, of the bell. Contraction is registered upwards in the upper trace and downwards in the lower trace. Electrical stimuli on the margin at *A* (lower trace) produce a response of the muscle propagated to *B* (upper trace). But the radial muscle at *A* then contracts, as shown by the maintained contraction on which is superimposed the local response of circular muscle (at *C*). Now the propagation to *B* is inhibited and the top line (at *D*) shows nothing. The contractions at *B* are an example of temporal summation in Hydromedusae.

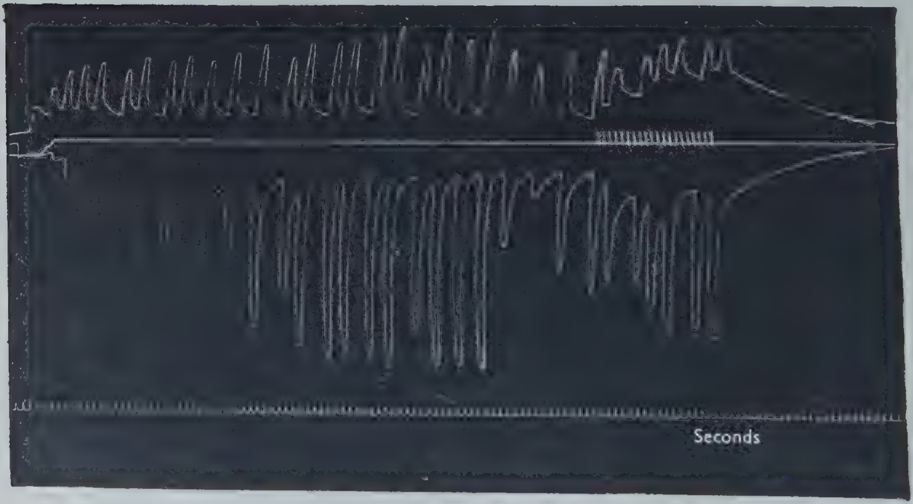


Fig. 4. A series of spontaneous beats recorded from opposite sides of the undamaged bell. Contraction is registered upwards in the upper trace and downwards in the lower trace. Twenty electrical stimuli applied at the place from which the upper record was taken produce a raising of the base-line as the radial muscle contracts, and the rhythm presently comes to an end.

conduction to the circular muscle over the subumbrellar epithelium. Fig. 3 is a record from two adjacent regions of the bell about 10 cm. apart. Electrical stimuli applied to the margin at the region from which the lower trace was recorded result in twitch-like contractions of the circular muscle in this region (*A*) which are also propagated to the adjacent region (*B*). After the tenth stimulus the radial muscle of the stimulated region develops a maintained contraction, on which the twitches of the circular muscle are superimposed (*C*). However these twitches are no longer propagated to the adjacent region (*D*). The significance of these observations is discussed below.

DISCUSSION

It has been shown that during the feeding reaction, groups of radial muscle are co-ordinated by a conducting system that runs predominantly radially, and that this system is physiologically distinguished from that co-ordinating the beat. A similar sharp distinction was found in *Geryonia* in which it was shown (Horridge, 1955) that the pointing reaction of the manubrium was unimpaired while the contraction wave was propagated continuously round the bell. However, in the normal *Aequorea*, or parts with the marginal nerves complete, contractions of the radial and circular muscles do not occur simultaneously. This does not arise from any property of the epithelial conducting elements or of the muscles themselves, for simultaneous contractions of both sets of muscles follow electrical stimuli (Fig. 4, *C*).

The inhibition of both rapid conduction and spontaneous rhythm when the radial system is active depends on some process in the margin, most likely in the ring nerves, which have ganglion cells all along their course. In inhibited sectors propagation of excitation to the circular muscle resembles that in sectors where the marginal nerves have been removed; an excitation is then transmitted only by slow pathways in the subumbrellar epithelium. The result is that during the feeding reaction the bell stops beating, at least in part and sometimes entirely. This observation is of some interest since it is to date the clearest example of inhibition in a coelenterate nervous system.

Aequorea is not the only medusa which shows an inhibition of the beat when the radial muscle contracts. The same observation has been made in the following genera: *Bougainvillea*, *Cunina*, *Melicertum*, *Mitrocoma*, *Oceanea*, *Phialidium*, *Podocoryne*, *Rathkea*, *Rhopolonema*, *Sarsia*, *Solmaris*, *Steenstrupia* and *Neoturris*, but cannot be conveniently recorded from small species.

In *N. pileata* a spontaneous beat was never observed in a region where radial muscles contracted locally, but a spontaneous beat in another region affected all the circular muscle, which also would respond to a single electrical stimulus. In this case spontaneous activity is inhibited but not rapid conduction. In other medusae, e.g. *Geryonia*, there is no inhibition of either kind. These differences between genera seem to be related to the type of movement involved in the feeding response, e.g. in *Aequorea* the margin bends inwards and the manubrium is displaced laterally towards it whereas in *Geryonia* there is no local bending of the margin, which can easily be reached by the long pseudomanubrium of this genus.

A further point of interest arises out of the consideration of the inhibition. In normal swimming the circular muscles contract regularly with no accompanying contraction of the radials. The pathways to the radial muscles are not inhibited, for mechanical stimulation will arouse a feeding response that in its turn inhibits the beat. It seems that the physiological pathways from the radial system to the rhythmical can act in one direction only. The same unidirectional path at this point of interaction between radial and rhythmical systems was noted in *Geryonia*, and a similar state of affairs is found in Scyphozoa. For example, in *Aurellia*, the marginal tentacles do not contract at each beat but stimulation of one of them may initiate a contraction wave in a preparation containing a tentaculocyst (Romanes, 1877).

SUMMARY

1. The co-ordination of the swimming movement (beat) and feeding response in *Aequorea forskalea* has been studied with particular attention to the pathways taken by the excitation.

2. The rapid through-conducting system which co-ordinates the beat is sharply distinguished physiologically from the radial system which co-ordinates feeding.

3. The spontaneous origin of the beat and its rapid marginal conduction are both inhibited while the radial muscle is contracting.

4. Inhibition of this type has been observed in other genera of Hydromedusae,

For help with the apparatus and provision of material, I am very pleased to express my gratitude to Dr R. Dohrn and his staff at the Stazione Zoologica, Naples, where this work was carried out during tenure of a grant from the Department of Scientific and Industrial Research.

REFERENCES

HERTWIG, O. & HERTWIG, R. (1878). *Das Nervensystem und die Sinnesorgane der Medusen*, 186 pp. Leipzig.

HORRIDGE, G. A. (1955). The nerves and muscles of medusae. II. *Geryonia proboscidalis*. *J. Exp. Biol.* (in the Press).

KRASINSKA, A. (1914). Beiträge zur Histologie der Medusen. *Z. Wiss. Zool.* **109**, 256.

MAYER, A. G. (1910). *Medusae of the World*. Carnegie Institute of Washington Publication no. 109, 3 vols. 735 pp.

PANTIN, C. F. A. & VIANNA DIAS, M. (1952). Rhythm and after-discharge in medusae. *An. Acad. bras. Cienc.* **24**, 351.

ROMANES, G. J. (1877). Further observations on the locomotor system of medusae. *Phil. Trans.* **167**, 659.

THE ROLE OF THE HAEMOCYTES IN THE GROWTH AND MOULTING OF AN INSECT, *RHODNIUS PROLIXUS* (HEMIPTERA)

By V. B. WIGGLESWORTH
Department of Zoology, University of Cambridge

(*Received* 30 *March* 1955)

Growth and moulting in insects is initiated by a hormone produced in the neurosecretory cells of the brain. This brain factor activates the thoracic (or 'prothoracic') glands, which are then believed to secrete the definitive 'growth and moulting hormone'. These conclusions are based chiefly on experiments with *Rhodnius* (Wigglesworth, 1940, 1952*a*), Lepidoptera (Fukuda, 1944; Williams, 1947), Diptera (Possompès, 1953) and Megaloptera (Rahm, 1952), and there is abundant histological and experimental evidence from other groups which points in the same direction (see Wigglesworth, 1954). The possibility remains, however, that there may be yet further links in the chain of endocrine organs, located, for example, in the abdomen. The object of the present work was to investigate this possibility in *Rhodnius*.

The chief organs in the abdomen in *Rhodnius* are the gut and Malpighian tubes, the heart and pericardial cells, the gonads, muscles, nerves and sense organs, the fat body, dermal glands, oenocytes and the haemocytes of various kinds. The gonads are not necessary for growth; the oenocytes and the dermal glands reach the height of their secretory activity long after growth in the epidermis has begun (Wigglesworth, 1933, 1948) and may therefore probably be excluded. In the present work attention has been concentrated on the pericardial cells and haemocytes. These tissues cannot readily be removed by surgical means. The experiments have consisted in observing the effects of injected substances which are specifically taken up by the cells in question—on the assumption that cells laden with such substances may thereby be 'blocked' and precluded from performing their other functions.

Most of the experiments have been done on the 4th-stage larva of *Rhodnius*. Injection is effected by holding the insect down with plasticene, cutting through the tibia of one of the hindlegs, inserting the abruptly tapered end of a graduated capillary tube, and forcing in by compressed air a measured volume of fluid. The capillary pipette is withdrawn while the tibia is compressed with forceps and the cut end sealed with paraffin wax. In this way known volumes can be injected without loss and with no mortality.

INJECTION OF TRYPAN BLUE, INDIAN INK AND IRON SACCHARATE

Fourth-stage larvae at 1 day after feeding were injected with 5 mm.^{3} of 0·02% trypan blue in Ringer's solution. Some were fixed with Carnoy's fluid 6 days later: the pericardial cells were filled with masses of the dye but there was none in the haemocytes. Mitosis was actively in progress in the epidermis. Those not dissected moulted at the usual time, 13–14 days after feeding. This small amount (1 μg.) of trypan blue is wholly taken up by the pericardial cells and causes no delay in moulting.

When 4th-stage larvae at 1 day after feeding were injected with 5 mm.^{3} of 0·2% trypan blue (10 μg.) and dissected 7 days later, not only were the pericardial cells filled with the dye, but the haemocytes were massed together in great clumps and within these cells the dye was segregated into vacuoles. There was no sign of mitosis in the epidermis, where it normally begins 4 days after feeding. At 15 days there was still no mitosis in the epidermis; the haemocytes had increased enormously in number. At 21 days mitosis had begun and moulting was in progress; it would probably have been completed about a week later. There was therefore a delay of about 2 weeks in the onset of moulting.

The results with trypan blue suggest that blockage of the pericardial cells alone does not affect moulting, but blockage of the haemocytes causes delay or temporary inhibition. This has been confirmed by the injection of indian ink, which is taken up only by the haemocytes. Stick ink was made up in boiled Ringer's solution to give a suspension of a density suitable for pen drawing, and 5 mm.^{3} were injected into 4th-stage larvae 1 day or 2 days after feeding. Among twelve insects the time required for moulting ranged from 21 to 50 days.

A third substance injected was iron saccharate, a 2% solution freshly prepared in Ringer's solution being used. This is taken up by the pericardial cells and by the haemocytes. 5 mm.^{3} injected at 1 day after feeding will delay moulting for 1–3 weeks beyond the normal 14 days.

EFFECT OF BLOCKING THE HAEMOCYTES AT DIFFERENT TIMES AFTER FEEDING

It is clear from the foregoing results that blockage of the haemocytes with trypan blue, indian ink, or iron saccharate will delay moulting. Is this effect due to interference with the general processes of intermediary metabolism, or to some specific action upon the initiation of mitosis and moulting in the epidermis?

This has been tested by injecting the substances at different times after feeding. Fig. 1 shows the effect of blockage of the haemocytes with iron saccharate at each day after feeding, from the first to the eighth. The average time required for moulting (at 25° C.) is 14 days. When injections were made on the first, second or third days after feeding, there was a delay ranging from 1 to 3 weeks. But injection on the fourth day and later caused no delay whatever. (There may be a slight delay of 24 hr. or so after the seventh or eighth day, due perhaps to the operation.)

Injection of an equivalent amount of Ringer's solution at 1 day after feeding caused no delay in moulting.

Injection of full-strength indian ink on successive days gave similar results. Batches of six larvae were injected as follows:

At 1 day after feeding: moulted at 20, 24, 30, 32, 37, 66 days
At 2 days after feeding: moulted at 19, 19, 21, 25, 35, 54 days
At 3 days after feeding: moulted at 29, 33, 39, 41, 78 (1 never moulted)
At 4 days after feeding: moulted at 15, 15, 15, 15, 17, 17 days
At 5 days after feeding: moulted at 15, 15, 15, 15, 15, 17 days

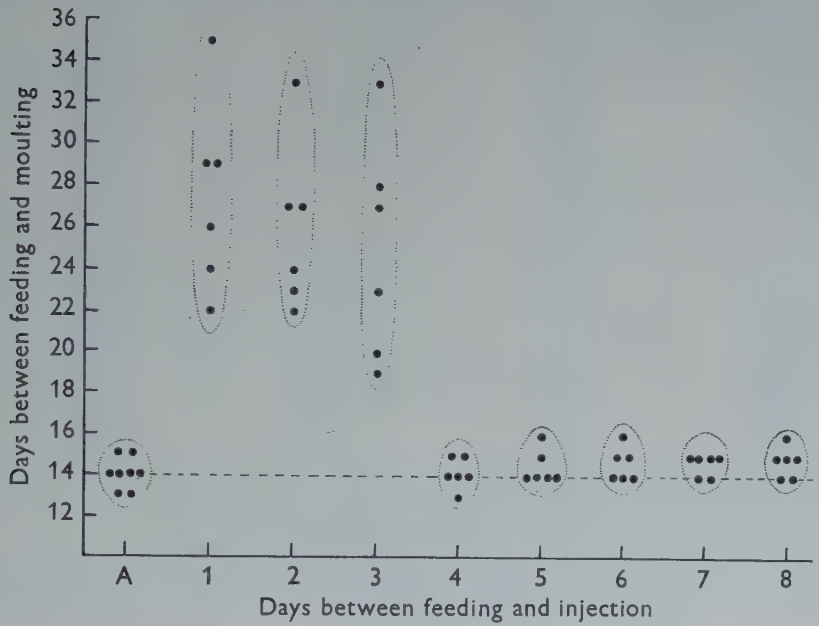


Fig. 1. Effect of blockage of the haemocytes on the time between feeding and moulting in the 4th-stage larva of *Rhodnius*. 5 mm.^{3} of 2 % iron saccharate was injected into batches of six larvae on the first to the eighth day after feeding. A, control batch of eight which received no injection.

Fig. 2 shows an experiment in which 5 mm.^{3} of a weak cloudy suspension of indian ink was injected: delay in moulting was very slight, but once more it was sharply limited to the first 3 days after feeding.

Clearly there is an abrupt change of some kind between the third and fourth days. The fourth day is the day on which mitosis in the epidermis usually begins and the visible process of moulting can be said to have started. It would appear that blockage of the haemocytes acts specifically upon the initiation of growth.

'CRITICAL PERIODS' IN THE MOULTING PROCESS

Moulting in *Rhodnius* can be prevented or delayed by a number of different procedures: (i) Removal of the neurosecretory cells of the brain by decapitation (Wigglesworth, 1934, 1940). (ii) Exclusion of the thoracic gland by isolation of the abdomen (Wigglesworth, 1952*a*). (iii) Exposure to temperatures above 35° C. (Wigglesworth, 1952*b*). (iv) Blockage of the haemocytes. Each of these procedures inhibits (or delays) moulting only when carried out before a certain 'critical period'. In the hope of throwing further light on these processes the critical period in each type of experiment was compared, using batches of twelve insects from the same culture, kept in the same incubator at 25° C. The results are shown in Fig. 3 and may be summarized as follows.

Decapitation (Fig. 3A). Of those decapitated 1 and 2 days after feeding, none moult. By 3 days after feeding, 80% moult; that is, most of the insects have already secreted sufficient of the brain factor to activate the thoracic glands. By 4 days, 100% moult.

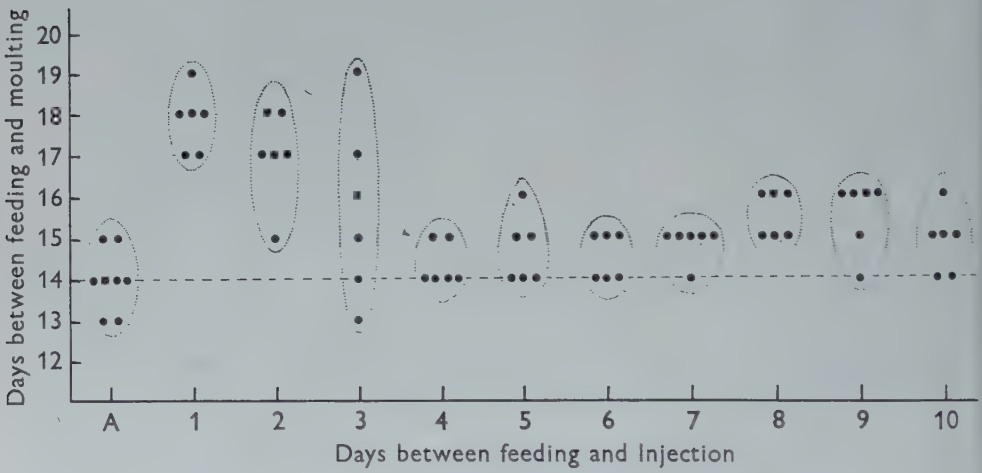


Fig. 2. Effect of injection of 5 mm.^{3} of very dilute indian ink into 4th-stage larvae of *Rhodnius*. Details as in Fig. 1.

Isolated abdomen (Fig. 3B). By 3 days, although 80% have liberated sufficient activating factor from the brain (see Fig. 3A), only 20% have secreted enough thoracic gland hormone for the isolated abdomen to moult. By 4 days, moulting of the isolated abdomen is still limited to 40%. By 5 days, 100% moult.

Thus nearly 2 days are required between the liberation of an adequate quantity of the activating secretion from the brain and the establishment of independence in the abdomen: by the end of 3 days the brain can be dispensed with in 80%; but not until the end of 5 days can the thorax be dispensed with in 100%.

Blockage of the haemocytes (Fig. 3C). In the blockage of the haemocytes there is a very sharp 'critical period' between the end of the third and the end of the fourth days. At the end of 3 days 100% show delayed moulting: in none have the haemocytes accomplished their task. At this time 80% can moult without the brain (Fig. 3A). The haemocytes cannot therefore be necessary for the secretion of the brain factor.

At this same time the isolated abdomen can moult in 20% (Fig. 3B). In a preliminary note (Wigglesworth, 1955*a*) this was taken as evidence that activation of the thoracic gland is independent of the haemocytes and that these must therefore intervene after the thoracic gland hormone has been liberated. Further experiments (see below) have shown that this conclusion was not justified.

Transfer to 36° C. (Fig. 3D). The 'critical period' for transfer from 25 to 36° C. does not agree exactly with any of the other three, but it comes closest to the critical period for moulting in the isolated abdomen. That would suggest that although the high temperature may well have an adverse effect on the secretion of the brain factor (for example, the thoracic glands show no signs of secretory activity in larvae kept at 36° C. from the time of feeding), it can also arrest moulting by interfering with the secretion of the thoracic gland hormone or with its action upon the epidermis.

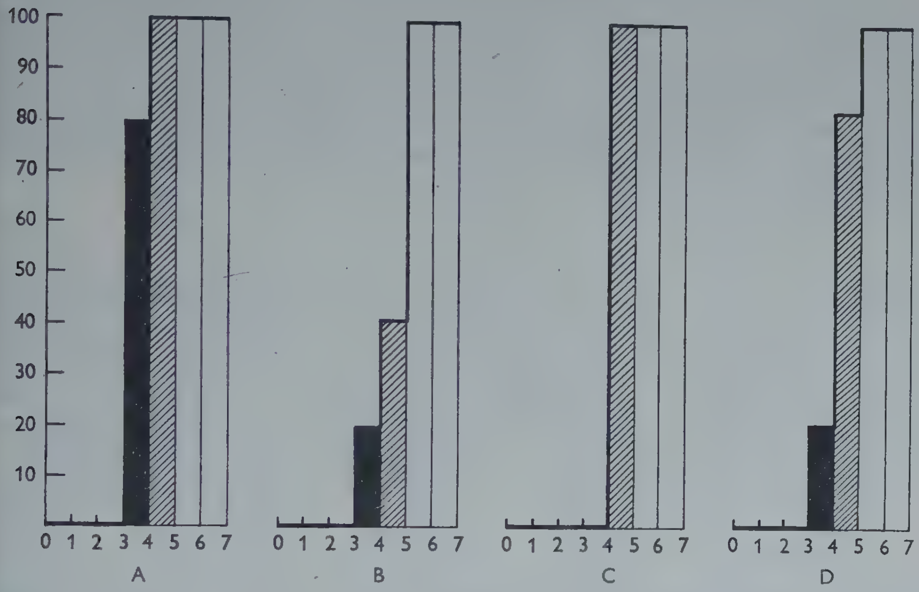


Fig. 3. 'Critical periods' for moulting in 4th-stage larvae of *Rhodnius* subjected to different experimental treatments. Ordinate: percentage of larvae moulting. Abscissa: day of operation. A, decapitation; B, isolation of abdomen; C, injection of iron saccharate or indian ink; D, transfer from 25 to 36° C.

This conclusion is supported by the observation that the 4th-stage larva, decapitated at 6 days after feeding, will induce moulting in another 4th-stage larva, decapitated at 1 day after feeding, to which it is joined in parabiosis at 25° C.—but if the insects after joining are placed at 36° C., although the larva decapitated at 6 days will moult, it will not induce moulting in its partner.

In general, these comparisons of the timing of the 'critical periods' as determined by different operative procedures support the conclusion that the haemocytes are concerned in one of the later steps in the hormonal initiation of moulting; but it is not possible from them alone to decide whether the haemocytes are involved in the production of the thoracic gland hormone or in the action of this upon the epidermis.

THE SITE OF ACTION OF THE HAEMOCYTES

Histological observations

The cells of the thoracic gland show a cycle of secretory activity during moulting (Wigglesworth, 1952*a*). In the 4th-stage larva at 1 day after feeding the nuclei are relatively small and the cytoplasm shrunken and inconspicuous (Fig. 4A). By 4 days after feeding the nuclei have become enlarged and stain more deeply and they are surrounded by more or less dense cytoplasm which is vacuolated at the periphery. Large numbers of haemocytes are closely associated with the thoracic gland cells (Fig. 4C).

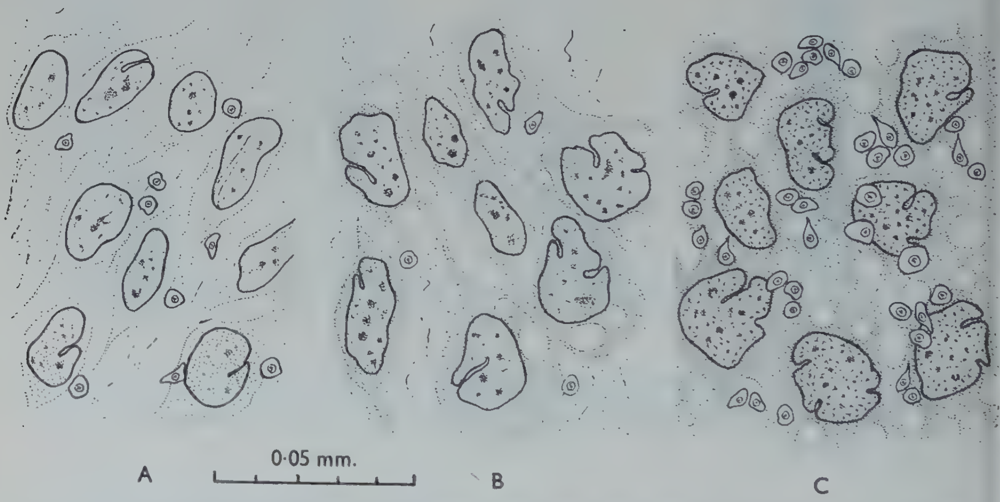


Fig. 4. A, part of the thoracic gland of 4th-stage larva 1 day after feeding. B, the same, 4 days after feeding in a larva injected with 5 mm.^{3} of 2% iron saccharate at 1 day; very few haemocytes present. C, the same, 4 days after feeding in normal larva; large numbers of haemocytes present.

In 4th-stage larvae injected with iron saccharate (5 mm.^{3} of 2% solution at 1 day after feeding) the nuclei have become similarly enlarged and lobulated by 4 days after feeding. There has also been some increase in the vacuolated cytoplasm around them; but both nuclei and cytoplasm stain far less deeply than in the normal insect (Fig. 4B). Haemocytes are almost absent (see p. 658).

These observations suggest that although there is some activation of the thoracic gland cells even when the haemocytes are blocked, the haemocytes are necessary if the cells are to develop their full secretory activity.

Experimental results

(i) Active thoracic glands were removed from 5th-stage larvae at 10 days after feeding. One gland from each pair was implanted into a decapitated 4th-stage larva (1 day after feeding) which had just been injected with 5 mm.^{3} of 2% iron saccharate; the other gland of the pair was implanted into a similar 4th-stage larva which had received no iron saccharate.

A single gland proved to be rather too small a dose, and only about 30% of the insects moulted. But there was no difference between the two groups: four out of twelve survivors in the control group moulted, and three out of ten survivors in the injected group; and moulting began at about the same time in all of them. It would appear that moulting induced by the implantation of a fully active thoracic gland is not delayed by blockage of the haemocytes.

(ii) The converse experiment consisted in implanting into the decapitated 4th-stage larva a pair of thoracic glands removed from a 5th-stage larva, 10 days after feeding, into which 10 mm.^{3} of 2% iron saccharate had been injected at 1 day after feeding. As a control, similar 4th-stage larvae received a pair of thoracic glands taken from normal 5th-stage larvae 10 days after feeding.

In the control series there were twelve survivors of which nine were induced to moult. Among those receiving thoracic glands from the larvae with blocked haemocytes, there were ten survivors of which only one moulted. Blockage of the haemocytes with iron saccharate has prevented the development of full secretory activity in the thoracic glands.

HISTOLOGICAL CHANGES IN THE HAEMOCYTES

A brief account of the haemocytes in *Rhodnius* has already been published (Wigglesworth, 1933), and a more detailed study will be published elsewhere; but a short description is necessary here. For the most part the cells are not floating freely in the circulating haemolymph, but are loosely adherent to the basement membranes. The following four types have been recognized.

(i) *Proleucocytes.* Small rounded forms with only a thin layer of darkly staining cytoplasm around the nucleus; non-phagocytic; often dividing; almost certainly the precursors of the amoebocytes and perhaps other types.

(ii) *Amoebocytes.* Highly pleomorphic; rounded, spindle-shaped, irregular or spread out in an attenuated form so as to merge imperceptibly into the basement membrane; usually furnished with numerous filamentous pseudopodia. These cells contain rounded, oval or rod-like deposits of mucopolysaccharide; they provide the substance of the basement membranes and connective tissue sheaths which have the same staining reactions and histochemical properties (Wigglesworth, 1955*b*). The amoebocytes are actively phagocytic.

(iii) *Oenocytoids.* Rounded or oval disk-shaped cells with small flattened nuclei. The cytoplasm is clear and homogenous or contains very minute vacuoles; the cell membrane sharply defined and refractile with no pseudopodia. The oenocytoids are non-phagocytic.

(iv) *Lipocytes.* Cells resembling small fat-body cells lying free in the body cavity. They are non-phagocytic and contain deposits of fat, glycogen and protein like the main fat-body cells.

In addition to these, two further types can be distinguished.

(v) *Large granular cells.* May be rounded or oval, but more often spindle-shaped. Nucleus large and vesicular with a very large round nucleolus; the cytoplasm filled with granules or small vacuoles.

(vi) *Large non-granular cells.* Usually spindle-shaped and often drawn out into filaments at either pole; resemble the large granular cells but the cytoplasm more or less hyaline.

Types (v) and (vi) are relatively very few in number; they are connected to the amoebocytes (ii) by intermediate types. But they lack the mucopolysaccharide deposits characteristic of the amoebocytes and they are either non-phagocytic, or very feebly phagocytic, for particles of indian ink or iron saccharate.

Since moulting is delayed by the injection of materials that are taken up by the phagocytic cells we are interested here chiefly in the *amoebocytes.* They are best studied by cutting along the margins of the abdomen and removing the tergites and sternites with the underlying heart, muscles, fat body and tracheal system *in situ.* These parts may be mounted fresh in Ringer's solution containing suitable vital dyes, or fixed with Carnoy's or Bouin's fixatives and mounted whole after staining for 3 min. with Prenant's ferric trioxyhaematein; or they may be cut, preferably in tangential sections, and stained in various ways. During the days which follow feeding, mitotic figures are plentiful among the proleucocytes, and the haemocytes of all types increase enormously in number.

Fig. 5A shows typical amoebocytes at 3 days after feeding when stained supravitally with gentian violet in Ringer's solution and mounted fresh. The cytoplasm shows a faint diffuse pinkish blue staining; it contains rounded or rod-shaped inclusions staining a slate-blue colour; there may be occasional clear vacuoles.

Fig. 5B shows amoebocytes similarly treated at 4 days after feeding. The average size of the cells is increased. In very many of them the cytoplasm is now almost completely filled with clear non-staining vacuoles, the blue-staining deposits being compressed between them. There are still some amoebocytes which are not vacuolated, but the difference between the majority of amoebocytes at 3 and 4 days after feeding is very striking. Similar vacuolated cells can be seen as late as 7 days after feeding at least.

Fig. 6A and B show typical amoebocytes on the third and fourth days after feeding, as seen in sections fixed with Bouin's fixative and stained with Masson's trichrome stain. In the fixed preparations the cells are smaller and the filamentous pseudopodia are not well preserved. At 3 days the nuclei stain red, the cytoplasm has a finely granular mauve colouring and contains rounded or rod-shaped deposits staining bright green. A few of the amoebocytes contain a single small vacuole. At 4 days the cytoplasm stains quite a deep purple but is often vacuolated and many cells are completely filled with vacuoles with the green-staining deposits appearing flattened between them.

Fresh preparations treated with other vital dyes and whole mounts and sections variously stained all give the same result: there is a striking change in the amoebocytes between 3 days (when injection of indian ink etc. delays moulting) and 4 days (when moulting is no longer delayed by injections).

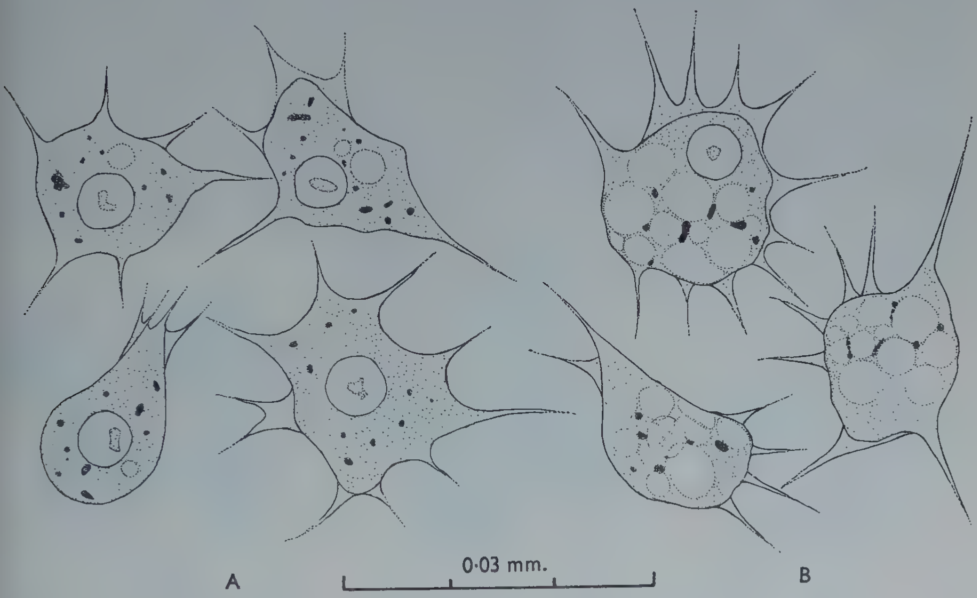


Fig. 5. A, typical amoebocytes in 4th-stage larva of *Rhodnius* at 3 days after feeding; supravital staining with gentian violet. B, the same at 4 days after feeding.

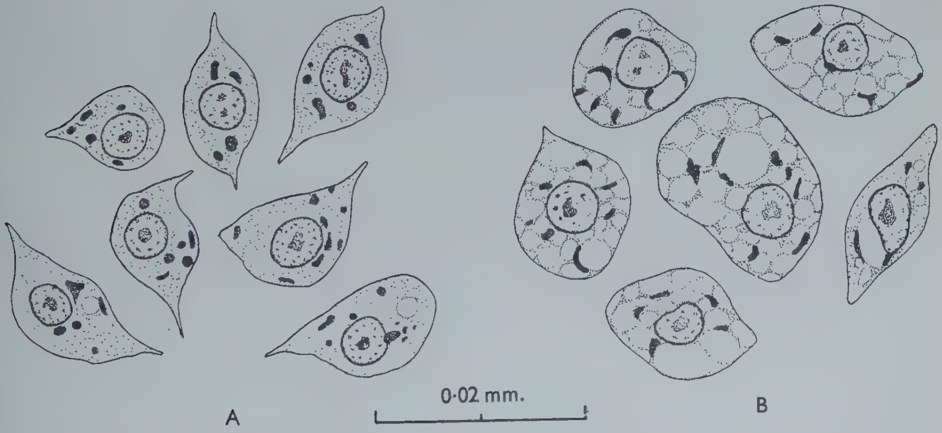


Fig. 6. A, typical amoebocytes in 4th-stage larva of *Rhodnius* at 3 days after feeding; from section fixed with Bouin's fixative and stained with Masson. B, the same at 4 days after feeding.

CHANGES IN THE HAEMOCYTES AFTER INJECTION OF IRON SACCHARATE AND INDIAN INK

Fourth-stage larvae were injected with the standard quantity of 5 mm.^{3} of 2% iron saccharate at different times after feeding. Then 1 day later the tergites and sternites were dissected off, fixed in Bouin's fixative and stained for iron by immersion in ammonium sulphide and subsequent treatment with 2–4-dinitrosoresorcinol (Humphrey, 1935), and counterstained with Prenant's ferric trioxyhaematein.

After injection at 1 day after feeding the amoebocytes are no longer dispersed over the surface of the various organs but are massed in clumps containing 100–500 or more contiguous cells. These cells are filled with iron deposits, mostly in the form of rounded droplets (Fig. 7A). A few of the cells are breaking down with the liberation of iron-containing droplets and little spheres of cytoplasm. The proleucocytes and oenocytoids contain no iron deposits.

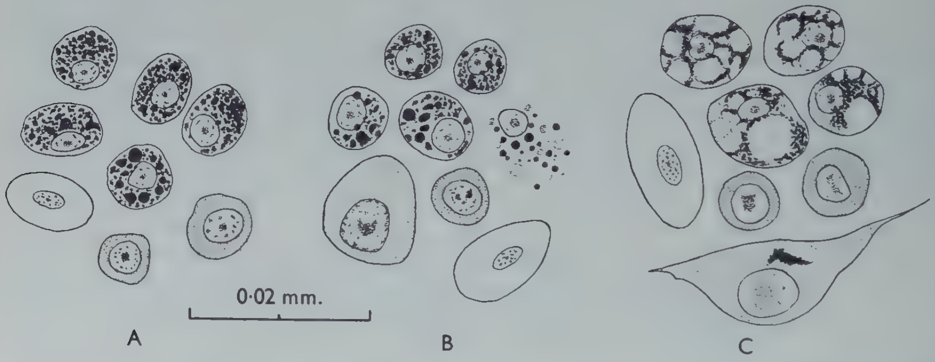


Fig. 7. Haemocytes in 4th-stage larva of *Rhodnius* at 1 day after injection with iron saccharate; dinitrosoresorcinol and haematoxylin. A, iron injection at 1 day after feeding: five amoebocytes filled with iron deposits, an oenocytoid and two proleucocytes. B, iron injection at 3 days after feeding: four amoebocytes filled with iron deposits, one amoebocyte breaking down, one large hyaline haemocyte, proleucocyte and oenocytoid below. C, iron injection at 4 days after feeding: four amoebocytes with iron deposits around vacuoles; oenocytoid to left, two proleucocytes and a large spindle-shaped haemocyte with a small deposit of iron.

After injection at 3 days the amoebocytes are massed as before and are filled with similar deposits of iron (Fig. 7B). Many more of these cells are now disintegrating and setting free cytoplasmic droplets. The figure shows an oenocytoid (now enlarging somewhat), a proleucocyte and a non-phagocytic giant haemocyte.

After injection at 4 days the amoebocytes are again mostly aggregated, though a larger proportion remains scattered. They are larger than at 3 days and appear to contain less iron. This is deposited in crescentic form over the surface of the clear vacuoles which more or less fill the cells (Fig. 7C). Occasional cells can be seen to be breaking down—but far fewer than after injection at 3 days. The oenocytoids are perhaps becoming still further enlarged. The figure also shows a large spindle-shaped haemocyte with a small deposit of iron.

After injection at 5 and 6 days the haemocytes are more scattered and show less tendency to form clumps. Vacuolated cells are present but are not so evident as at 4 days. Few of the cells are breaking down.

Similar results are obtained after the injection of indian ink. When injected at 2 days after feeding, and fixed 1 day later the amoebocytes are so densely packed with spherical droplets filled with the ink that even the nuclei are barely visible (Fig. 8A). Aggregation into clumps occurs, but is not so evident as after the injection of iron saccharate.

When injected at 3 days, many of the cells are filled with the ink as at 2 days; but there are more cells which are not quite so densely packed with the ink droplets (Fig. 8B). There is very little break-down among the cells such as occurs after the injection of iron.

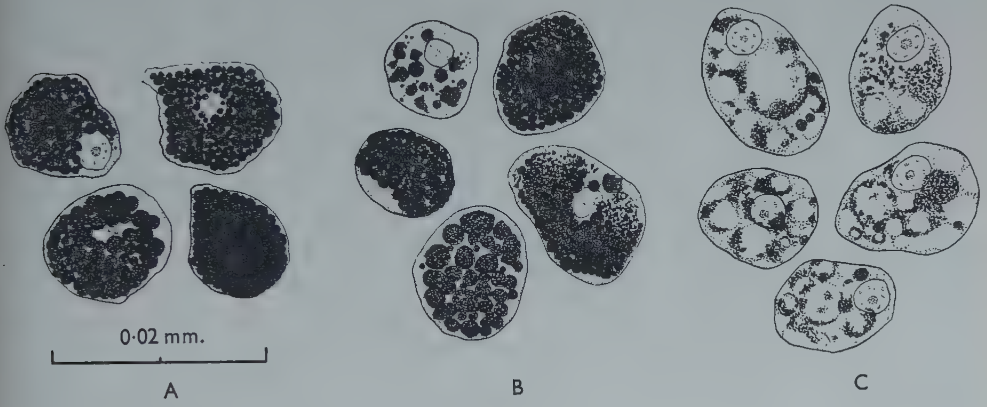


Fig. 8. Amoebocytes in 4th-stage larva of *Rhodnius* at 1 day after injection with indian ink. A, injection 1 day after feeding. B, injection 3 days after feeding. C, injection 4 days after feeding, showing indian ink particles over the surface of vacuoles.

After injection at 4 days, the haemocytes are more scattered; they are slightly larger than at 3 days and contain much less indian ink. This is no longer densely packed but is diffuse and often separated by the vacuoles which fill the cells (Fig. 8C).

There are occasional vacuolated cells with relatively little ink after injection at 3 days, and occasional cells with dense deposits of ink at 4 days; but the figures and descriptions indicate the state of the majority of the cells present on these days. The results confirm the observations on the histology of the normal haemocytes in showing a striking change in the secretory activity of the amoebocytes between the third and fourth days after feeding.

THE RELATION OF THE HAEMOCYTE FACTOR TO THE CRYSTALLINE HORMONE OF BUTENANDT & KARLSON

Butenandt & Karlson (1954) have recently succeeded in isolating in crystalline form from pupae of the silkworm, an active principle which will induce puparium formation in *Calliphora* and which, as proved by Williams (Butenandt & Karlson,

1954) will bring about renewed development in the diapausing pupa of *Platysamia*. Through the kindness of the German authors I have been able to test the activity of their hormone on *Rhodnius*.

The hormone was made up in saline at a concentration of 50 μg. or 100 μg. per ml. This was injected, by the method outlined above, into 4th-stage larvae at 1 day after feeding. The larvae were then decapitated. The 4th-stage larva at this time weighs about 80 mg., most of this weight being due to the blood meal in the stomach. Doses ranging from 0·025 to 0·25 μg. were given to twelve insects, but all the results were negative.

A dose of 0·25 μg. was then given at 1 day after feeding and repeated at daily intervals for 3 days, making a total of 0·75 μg. The two larvae so treated were dissected at 5 days after feeding; mitosis was actively in progress in the epidermis.

This experiment was repeated on two further larvae, one of which received a total of 0·75 μg. of hormone, the other 0·5 μg. By 25 days both had completed the formation of the new cuticle and the digestion of the old. The new cuticle (as was to be expected in the absence of the corpus allatum) was of the adult type.

The active principle from the silkworm pupa will thus induce moulting in the decapitated *Rhodnius*. In his tests in *Platysamia* Williams found that diapause could be terminated and development induced by injection of the hormone into the isolated abdomen. This experiment also has been carried out on *Rhodnius*. Four 4th-stage larvae at 1 day after feeding were injected with a single dose of 1·0 μg. of the hormone and the abdomen immediately isolated by a ligature through the metathorax as previously described (Wigglesworth 1952*a*). All four were induced to start moulting, although they died or were dissected and fixed before the process was complete. Six larvae were given 0·75 μg. in a single dose and treated in the same way: one died, moulting was induced in all the remaining five.

Since their hormone was effective in the isolated abdomen Butenandt & Karlson (1954) suggested that it is probably the active principle of the thoracic gland. We have seen that moulting induced by implantation of the fully active thoracic gland is not delayed or inhibited by blockage of the haemocytes. It was therefore of interest to test the crystalline hormone in the same way.

With this object four 4th-stage larvae were decapitated at 1 day after feeding. Two of them were given a single dose of 1 μg. of the hormone and two were given the same dose of hormone plus 5 mm.^{3} of 2% iron saccharate. All started to moult at about the same time: the characteristic changes which become visible in the integument of the normal insect at 7 days after feeding had become apparent by 5 days, that is, by 4 days after the injection. Moulting was complete in 17–20 days.

This experiment was repeated with indian ink. Four 4th-stage larvae decapitated at 1 day after feeding were given 1 μg. of the hormone, and two of them received in addition 5 mm.^{3} of a strong suspension of indian ink. Moulting had clearly begun in all four insects by 6 days after feeding and went forward at approximately the same rate in all of them.

Thus, blockage of the haemocytes causes no delay in the onset of moulting induced by the crystalline hormone of Butenandt & Karlson. This agrees with the suggestion that this hormone is the factor normally produced by the thoracic gland.

The study of the 'critical period' for transfer from 25 to 36° C. in the 4th-stage larva (p. 653) suggests that the high temperature may not only arrest the secretion of the thoracic gland hormone but may interfere with the subsequent action of this hormone in the abdomen.

Four 4th-stage larvae were decapitated at 1 day after feeding and each was given a single injection of 1 μg. of hormone. Two were placed at 25° C. and two at 36° C. At 12 days after feeding moulting was well advanced in those at 25° C., and by 20 days both had moulted to the adult type. Those placed at 36° C. showed no sign of moulting at 12 days; at 20 days both had died but in neither had moulting commenced. This result confirms the earlier conclusion that high temperature prevents the initiation of mitosis in the epidermis, even when the necessary hormones are already present.

DISCUSSION

Throughout the study of growth hormones in *Rhodnius* it has always been maintained that the 'moulting hormone' might prove to be a complex of successive secretions. It would now appear that the neurosecretory cells in the brain, the amoebocytes, and the thoracic glands constitute a secretory chain necessary for the initiation of growth. But there is no reason to suppose that the story is yet complete. The oenocytoids and other non-phagocytic haemocytes are undergoing cycles of activity; the part they are playing in the intermediary metabolism of growth remains to be proved. The role of the pericardial cells is very incompletely understood. And it is possible that there may be some division of labour among the epidermal cells themselves.

How the amoebocytes intervene in the moulting process is not known. They might be concerned in transporting the hormone from the neurosecretory cells to the thoracic gland. But some degree of activation of the thoracic gland occurs even when the amoebocytes are blocked. It is perhaps more probable that they are secreting some raw material that is necessary for the full activity of the thoracic glands. Alternatively, it is possible that the brain factor is removed from the blood by the phagocytic activity of the amoebocytes; or, as Prof. C. M. Williams has suggested to me, it is conceivable that during their disturbed metabolism the amoebocytes might inactivate the brain factor in some other way.

The demonstration of the part played by the amoebocytes in moulting raises the question of their possible role in wound healing. The histological changes in wound healing in *Rhodnius* are the same as those in normal moulting (Wigglesworth, 1937). These changes can go forward in the decapitated insect. It was therefore suggested (Wigglesworth, 1954) that the epidermis might be responsible for the localized production of factors comparable with those produced centrally in the normal process of moulting. Now it is characteristic of wound healing that there is a large aggregation of haemocytes around the site of injury. It may be that they are concerned in this local process of hormone production. (It may also be that the large local increase in haemocytes after wounding may be concerned in the persistent rise in the respiration rate which Schneiderman & Williams (1953) have observed to follow such injuries.)

One further point calls for comment. Discussions on the function of the haemocytes have generally been based on the assumption that a given type of blood cell has a single function. It is quite clear that the amoebocytes in the blood of *Rhodnius* have several functions: they are active phagocytes; they produce the mucopolysaccharides which go to form the basement membranes and connective tissues (Wigglesworth, 1955*b*); and they produce the droplets of secretion which appear to be essential for the full activity of the thoracic gland. This multiplicity of function in a single cell is equally evident in the fat body cells and in the epidermal cells of insects.

SUMMARY

Moulting in *Rhodnius* is greatly delayed by the injection of trypan blue, indian ink or iron saccharate, which are taken up by the phagocytic blood cells (amoebocytes).

This delay occurs only when the injections are made during the first 3 days after feeding, not on the fourth day or later.

On the fourth day after feeding the amoebocytes become filled with secretory vacuoles which restrict the uptake of indian ink or iron saccharate.

When the amoebocytes are blocked by such injections within a day after feeding, the secretory activity of the thoracic gland is greatly reduced. This is proved histologically and experimentally. On the other hand, moulting induced by implantation of a fully active thoracic gland is not delayed by blocking the haemocytes.

It is therefore concluded that some substance secreted by the haemocytes is necessary for the full activity of the thoracic glands.

The crystalline hormone isolated by Butenandt & Karlson from the silkworm will induce moulting in the decapitated *Rhodnius* larva and in the isolated abdomen. Its action is not inhibited or delayed by the injection of indian ink or iron saccharate. This supports the conclusion that it is the product of the thoracic gland.

Sublethal high temperatures will inhibit moulting in *Rhodnius* even when it is provided with abundant hormone by injection of the crystalline material or by joining in parabiosis with a moulting larva.

I am indebted to Prof. A Butenandt and Dozent Dr P. Karlson of the University of Tübingen for a supply of their crystalline hormone.

REFERENCES

Butenandt, A. & Karlson, P. (1954). Über die Isolierung eines Metamorphose-Hormone der Insekten in kristallisierter Form. *Z. Naturf.* **9***b*, 389–91.

Fukuda, S. (1944). The hormonal mechanism of larval molting and metamorphosis in the silkworm. *J. Fac. Sci. Tokyo Univ.* Sect. iv, **6**, 477–532.

Humphrey, A. A. (1935). Dinitrosoresorcinol—a new specific stain for iron in tissues. *Arch. Path.* (*Lab. Med.*), **20**, 256–8.

Possompès, B. (1953). Recherches expérimentales sur le déterminisme de la métamorphose de *Calliphora erythrocephala* Meig. *Arch. Zool. exp. gén.* **89**, 203–364.

RAHM, U. H. (1952). Die innersekretorische Steuerung der postembryonalen Entwicklung von *Sialis lutaria* L. (Megaloptera). *Rev. suisse Zool.* **59**, 173–237.

SCHNEIDERMAN, H. A. & WILLIAMS, C. M. (1953). Metabolic effects of localized injury to the integument of the cecropia silkworm. *Anat. Rec.* **117**, 640–1.

WIGGLESWORTH, V. B. (1933). The physiology of the cuticle and of ecdysis in *Rhodnius prolixus* (Triatomidae, Hemiptera); with special reference to the function of the oenocytes and of the dermal glands. *Quart. J. Micr. Sci.* **76**, 269–318.

WIGGLESWORTH, V. B. (1934). The physiology of ecdysis in *Rhodnius prolixus* (Hemiptera). II. Factors controlling moulting and 'metamorphosis'. *Quart. J. Micr. Sci.* **77**, 191–222.

WIGGLESWORTH, V. B. (1937). Wound healing in an insect (*Rhodnius prolixus* Hemiptera). *J. Exp. Biol.* **14**, 364–81.

WIGGLESWORTH, V. B. (1940). The determination of characters at metamorphosis in *Rhodnius prolixus* (Hemiptera). *J. Exp. Biol.* **27**, 201–22.

WIGGLESWORTH, V. B. (1948). The structure and deposition of the cuticle in the adult mealworm, *Tenebrio molitor* L. (Coleoptera). *Quart. J. Micr. Sci.* **89**, 197–217.

WIGGLESWORTH, V. B. (1952*a*). The thoracic gland in *Rhodnius prolixus* (Hemiptera) and its role in moulting. *J. Exp. Biol.* **29**, 561–70.

WIGGLESWORTH, V. B. (1952*b*). Hormone balance and the control of metamorphosis in *Rhodnius prolixus* (Hemiptera). *J. Exp. Biol.* **29**, 620–31.

WIGGLESWORTH, V. B. (1954). *The Physiology of Insect Metamorphosis.* Monographs in Exp. Biol., no. 1. Cambridge University Press.

WIGGLESWORTH, V. B. (1955*a*). The endocrine chain in an insect. *Nature, Lond.*, **175**, 338.

WIGGLESWORTH, V. B. (1955*b*). The haemocytes and connective tissue formation in an insect *Rhodnius prolixus* (Hemiptera). *Quart. J. Micro. Sci.* (in the press).

WILLIAMS, C. M. (1947). Physiology of insect diapause. II. Interaction between the pupal brain and prothoracic glands in the metamorphosis of the giant silkworm, *Platysamia cecropia*. *Biol. Bull., Woods Hole*, **93**, 89–98.

IONIC REGULATION IN THE MUSCLE FIBRES OF *CARCINUS MAENAS*

II. THE EFFECT OF REDUCED BLOOD CONCENTRATION

By J. SHAW
Department of Zoology, King's College, University of Durham,
Newcastle upon Tyne

(*Received* 10 *March* 1955)

INTRODUCTION

It is now well known that the common shore crab, *Carcinus maenas*, is able to live for long periods of time in diluted sea water, and that in dilutions below about 70 or 80% the animals are able to maintain the total osmotic pressure of their blood well above that of the outside environment. Since it is inorganic ions, of which the most important, numerically, are sodium and chloride ions, which account for the greater part of the osmotic pressure exerted by the blood, this process of osmotic regulation is dependent on the underlying process of ionic regulation. It is, however, a noteworthy fact that although the total osmotic pressure of the blood is often greater than that of the outside medium in these conditions, it is very much lower than that of the blood of animals living in normal sea water. For example, if crabs are kept in 25% sea water until a steady state is reached, the blood osmotic pressure falls to a new value about 50% lower than that which is found in the normal animals. The mechanisms underlying the osmotic and ionic regulation of the blood of *Carcinus* are now fairly well understood, but little is known of the ways in which the cells and tissues of the animal are able to withstand and adapt themselves to such very large changes in the blood concentration. Such processes of cell adaptation must be widespread among marine animals which penetrate into brackish waters, since the blood osmotic pressure is rarely kept exactly constant, but these powers of adaptation seem to be lost in fresh-water animals.

Krogh (1939, p. 85) gives some preliminary analyses of some inorganic ions in the muscles of *Eriocheir* from sea water and from fresh water. He found a decrease in chloride concentration of 61·9% and smaller decreases for sodium and potassium —36·4 and 24·3% respectively—the latter in spite of the fact that the blood potassium concentration was apparently higher in the animals from fresh water. It is not certain to what extent these analyses represent true muscle concentrations, since the method of preparing the muscles free from blood are not given.

In order to shed some further light on this problem of cell adaptation, the experiments described in this paper were designed to investigate the changes in the concentrations of some inorganic ions which occur in one type of tissue—viz. the large striated muscle fibres—when the blood composition is altered as a result of a new equilibrium with diluted sea water.

In the first paper of this series (Shaw, 1955), it was shown on analyses of single muscle fibres from the carpopodite extensor and flexor muscles of the chela that inorganic ions accounted for only about one-third of the total osmotic pressure of the fibre if this was isosmotic with the blood. Of the ions which were studied only potassium and chloride appeared to be in equilibrium with the corresponding ions of the blood, and hence distributed in a passive manner across the fibre membrane. It seemed possible that the Donnan equilibrium theory, advanced by Boyle & Conway (1941) for frog muscle, might be applicable, also, to *Carcinus* muscle fibres. It was suggested that the fibres might also be permeable to sodium so that the theory would have to be modified to include a sodium-secreting mechanism as is the case for the vertebrate muscle (Dean, 1941; Krogh, 1946). The permeability of the crab muscle fibre to sodium has since been confirmed by the use of radioactive sodium as a tracer, and it was found that the whole of the muscle sodium was exchangeable. The quantitative aspects of this work will be presented in another paper.

The experiments described below show to what extent the theory of Boyle & Conway can account for the behaviour of the muscle fibres in diluted blood, and whether the ionic changes occurring in the muscle fibre are a result of normal diffusion processes setting up new equilibria or are mediated by active mechanisms.

MATERIAL AND METHODS

For the whole of this investigation freshly collected crabs were used and these were not fed during the course of the experiments. A batch of crabs was placed in an aquarium containing a relatively small quantity of sea water, and this was gradually diluted by letting diluted sea water of known strength drip into it and mix with it. The mixture left through an outflow, and the flow of dilute sea water was so adjusted that the normal sea water was replaced by the diluted sea water in a few days. The crabs were allowed to remain in this new solution for another 4 days in order that the new steady state had time to be established, and during this time the dilute sea water continued to drip into the aquarium to keep the water aerated and circulated. Only crabs which survived this treatment and were apparently normally active were used for subsequent analysis.

The analytical methods were the same as those described in the previous paper (Shaw, 1955) and, as before, only the carpopodite extensor and flexor muscles of the chela were used. Since it was found then that there was a very close correspondence between concentration of ions in single muscle fibres and in whole muscles prepared by careful washing, whole muscles were used exclusively for the work described here.

The method used for measuring the resting membrane potential was improved so that intracellular electrodes of higher resistance could be used. The new amplifier consisted of a GEC subminiature electrometer triode (type ET 3) connected in a cathode follower circuit. The operating conditions were such that the grid current was not greater than 10^{-14} A. and this allowed the use of a larger grid resistor ($10^{10}\ \Omega$). The valve and the grid resistor were mounted together on the arm of the micro-manipulator and the grid was connected by a short screened lead to the electrode. The increased input impedance of the amplifier circuit allowed the use of electrodes with finer tips—the average tip diameter was less than 1 μ and the resistance of the complete electrode between 10 and 50 MΩ.

BLOOD COMPOSITION

The variations in the blood composition following immersion in diluted sea water have been studied by Webb (1940) who has shown to what extent the concentrations of various inorganic ions are regulated by the animal. The measurements of blood composition made here do not extend these findings, but some of the analyses are presented as they display several features which call for special comment. Fig. 1 shows the relation between the chloride and potassium concentration of the blood and the dilution of sea water in which the animals were living. In the case of the chloride ion, at dilutions down to 70% the blood chloride falls in proportion, but at lower dilutions there is a plateau region which extends to a dilution of about 40% where the blood chloride remains almost constant (at about 350 mM./l.). At greater dilutions the level falls steeply once more and this can be correlated with the survival of the animals in the dilute sea waters. At dilutions down to 40% all the crabs generally survive, but as the dilution increases so the proportion of animals surviving decreases. The concentration of sodium in the blood (not shown in the

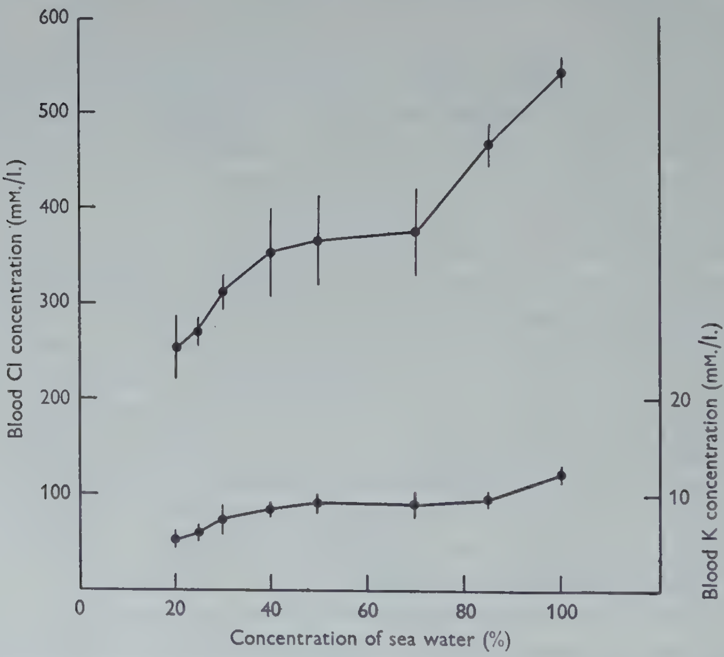


Fig. 1. The relation between the concentrations of chloride and potassium in the blood and the dilution of the outside sea water. The points are mean values; the vertical lines the standard deviations of six readings.

figure) follows that of the chloride quite closely except that at all dilutions it is somewhat lower.

Another noticeable feature is the great variability of the blood chloride concentration in the same batch of crabs, particularly in the plateau region, and which must be the result of very different powers of osmoregulation possessed by different individuals. Because of this great variability, in subsequent sections the properties of the muscle fibres such as the water content and the membrane potential are related to the blood chloride concentration rather than the dilution of sea water in which the animals were living.

The concentration of potassium in the blood is also shown in Fig. 1., and it can be seen that it follows the same sort of trend as the chloride ion but at a much lower level. There is an initial fall, then a plateau corresponding to a concentration of about 9 mM./l. and finally a steeper fall at dilutions below 40%.

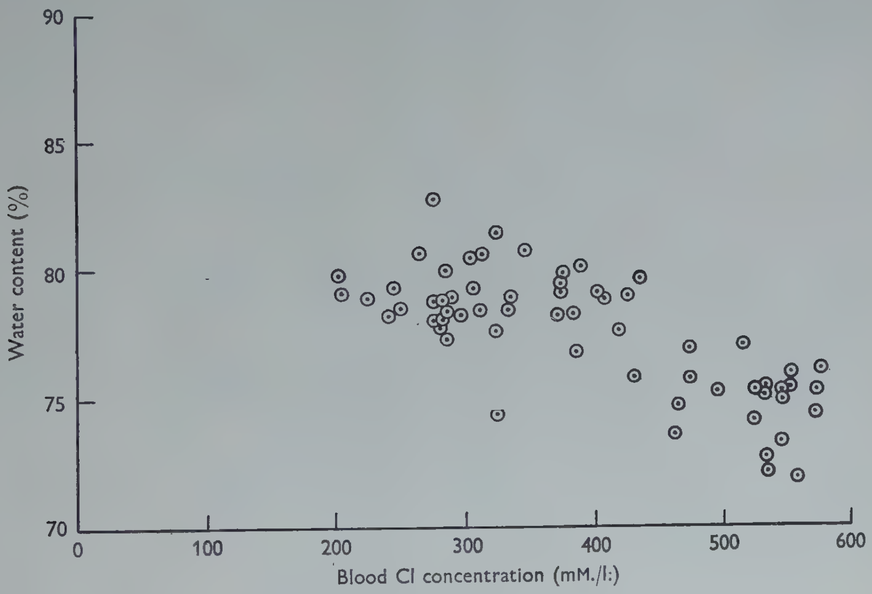


Fig. 2. The relation between the blood chloride concentration and the percentage water content of the muscles.

WATER CONTENT OF THE MUSCLES

For a study of the changes in the concentration of ions in the muscle fibres and their interpretation in terms of membrane equilibria it is of the greatest importance to know to what extent these changes are brought about by an increase in the water content of the muscle due to the swelling of individual fibres.

The total water content of all the muscles used for analysis was determined as the difference between the wet weight of the washed muscle and its weight after drying at 100° C. and is expressed as a percentage of the wet weight. Fig. 2 illustrates the relation between the percentage water content of the muscle and the chloride concentration of the blood. The mean value for the water content of the muscles of crabs from normal sea water is 74·5 % (eighteen measurements) and this gradually increases until a value of about 80 % is reached in the lowest concentrations. The mean value for crabs from 20 % sea water is 79·6 % (eleven measurements). This change in the water content of the fibres may be due to two causes. It may be the result of water entering the fibres and causing them to swell or it may be brought about by the loss of diffusible solids from the fibres or, as is probably the case, a combination of both. It is not, however, very easy to come to a decision as to the relative importance of the two processes, but we can readily estimate the maximum degree of swelling which could have occurred. Assuming that the whole of the change of water content is due to an intake of water and an increase in volume of the fibre, then the solids, which in the normal fibre must account for 25·5 % of the total weight of the fibre, are diluted so that they account finally (in the 20 % series) for only 20·4 %. This means there would be a fall in concentration of every substance in the muscle of about 20 % and the increase in muscle weight is exactly 25 %. This last figure represents the maximum degree of swelling, but is based on an average figure—greater volume increases would be possible in certain individuals.

No reliable estimate can be made of the extent to which solids are lost from the fibres. It will be shown below that the total loss of inorganic ions is probably quite small, but, on the other hand, the greater part of the osmotic pressure of the fibres is made up of un-ionized molecules. If the fibres are always in osmotic equilibrium with the blood, then there must be a great reduction in the osmotic activity of these un-ionized substances. The obvious method by which this could be done is by the removal of a proportion of the molecules from the fibre but, on the other hand, if these substances are largely free amino-acids it would be possible to reduce their osmotic activity by linking them together to form larger molecules, such as polypeptides or proteins. Clearly a detailed investigation of this side of the problem is required and all that can be said at the moment is that the evidence of the change of concentration of some of the inorganic ions, given below, suggests that swelling of the fibres does occur and that the degree of swelling is probably not much less than the maximum predicted value.

MEMBRANE POTENTIAL

The resting membrane potentials of the individual fibres of the carpopodite extensor muscle were measured in five crabs from each dilution of sea water and from each crab between ten and twenty muscle fibres were examined. For the crabs taken from normal sea water the mean value is 58 mV. and this is the same value as was found in previous measurements (Shaw, 1955) so that the use of the finer electrodes has not resulted in an increase in the recorded potential. The values of the membrane potential in the crabs from the higher dilutions are shown in Fig. 3 which relates the potentials to the blood chloride concentration. It is a striking fact that the membrane potential remains practically constant at 59 mV. (S.D. = ± 3 mV., $n = 32$) over the whole range of blood dilutions, and despite the fact that at the same time considerable changes in the blood potassium concentration are occurring. However, although it is well known that if solutions containing low potassium concentrations are applied to nerve and muscle, an immediate increase in the membrane potential results—and this has been shown to occur in crab muscle fibres (Fatt & Katz, 1953)—this effect is an instantaneous one and occurs before a steady state has been reached between the muscle and the surrounding solution.

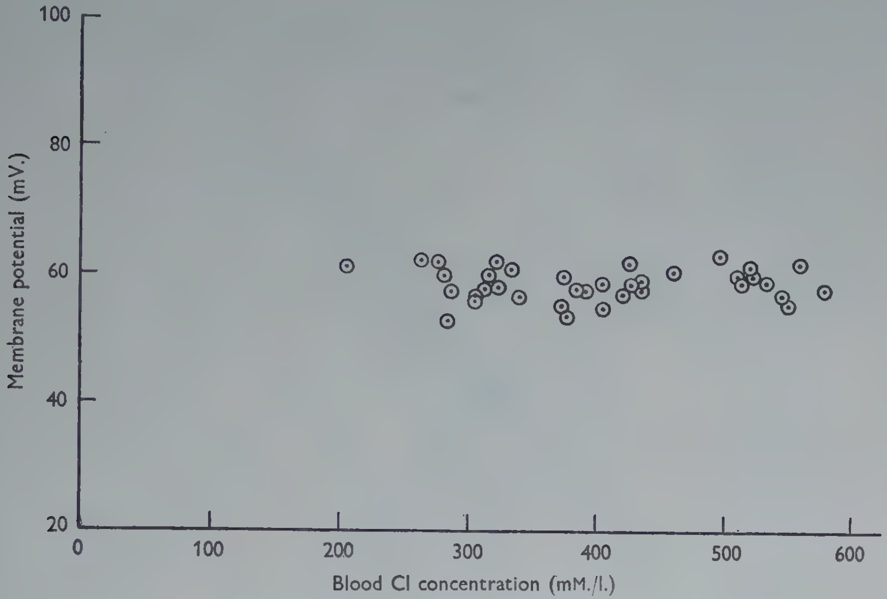


Fig. 3. The relation between the muscle fibre membrane potential and the blood chloride concentration.

CONCENTRATION OF INORGANIC IONS

(*a*) *Chloride*

In normal muscle fibres it was found (Shaw, 1955) that the chloride concentration was 50 mM./kg. fibre water and that in view of the existing membrane potential of 58 mV. this ion was in equilibrium with the chloride in the blood, since its concentration in the blood was roughly ten times that in the muscle. In muscles of crabs kept in diluted sea water the chloride content falls and the drop in concentration is greater than can be accounted for by the increase in water content of the muscle, even if all this is attributed to swelling of the fibres. There is no doubt that the muscle fibre membrane is permeable to chloride. The relation between the chloride concentration of the muscles (expressed as mM./kg. fibre water) and the blood chloride concentration is shown in Fig. 4. The straight line in the figure is the calculated regression line and the regression coefficient for the muscle chloride on the blood chloride is 0·102. This regression is highly significant, and there is therefore a constant ratio between the blood chloride and muscle chloride of about 10 which extends over the whole range of blood chlorides measured. The ratio

$Cl_o/Cl_i = 10$ corresponds to an equilibrium potential across the fibre membrane of 58 mV. at 18° C. and this is in such excellent agreement with the mean value of the resting potential of 59 ± 3 mV. that there seems little doubt that the redistribution of the chloride ion is brought about by normal diffusion processes.

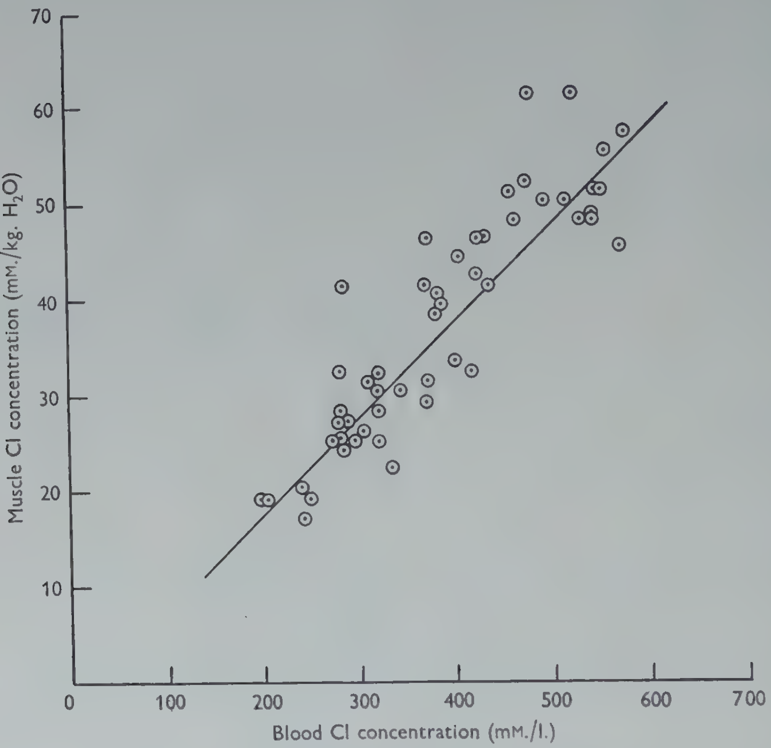


Fig. 4. The relation between the blood chloride concentration and the muscle chloride concentration.

(*b*) *Sodium*

The determination of the sodium ion concentration of the same muscles shows likewise a large fall in the concentration from the normal mean value of 50 mM./kg. fibre water. In the lower blood concentrations the sodium level falls to less than half this value, and, as with chloride, this decrease is much greater than could be caused by the maximum degree of swelling. Therefore sodium must have leaked out from the fibres and the muscle membrane must also be permeable to this ion—a fact that has been confirmed by the exchange of the muscle sodium with radioactive sodium injected into the blood.

The relation between the concentration of sodium in the blood and in the muscles is shown in Fig. 5. The scatter is greater than for chloride, but the correlation between the blood and muscle concentrations is a very highly significant one. The regression of the muscle sodium on the blood sodium is also shown in the figure and the regression coefficient is 0·0994; thus, the ratio Na_o/Na_i is also constant over the whole range and is approximately equal to 10, the same ratio found for the chloride ion. Since the equilibrium potential corresponding to this ratio (−58 mV. at 18° C.) is of opposite sign to the measured resting potential, the loss of sodium from the fibres must be brought about by an active secretion against the electrochemical gradient—a process requiring the performance of work by the muscle cell. This is clearly simply an extension of a process occurring normally in the muscle fibre, whereby the sodium ions diffusing into the fibre are pumped out again and a steady state achieved. This phenomenon seems generally applicable to

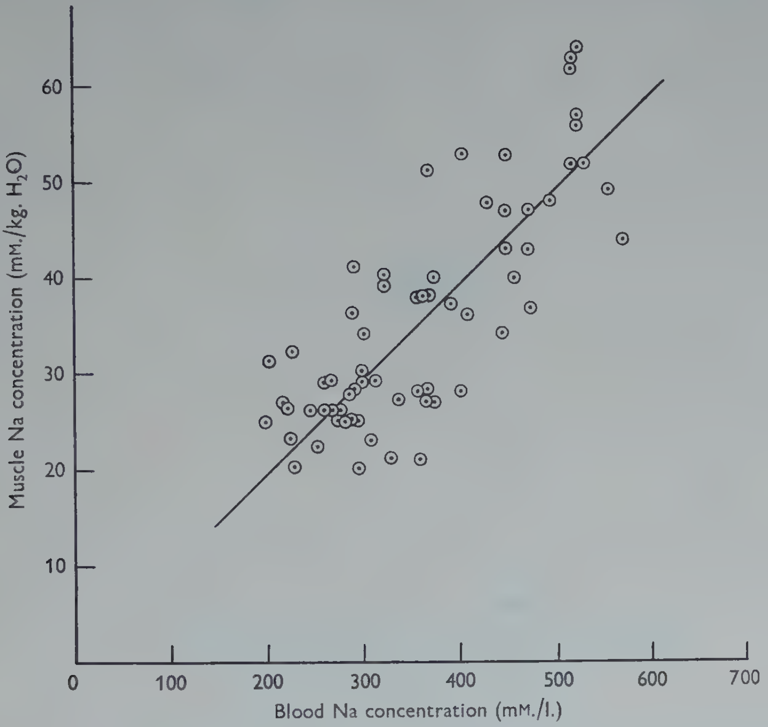


Fig. 5. The relation between the muscle sodium concentration and the blood sodium concentration.

muscle cells, and the permeability of vertebrate muscle cells to sodium has been repeatedly demonstrated (Levi & Ussing, 1948; Harris & Burn, 1949; Steinbach, 1951; Keynes, 1954). If the movement of sodium ions from the blood to the muscle is mediated by normal diffusion processes, then the rate of diffusion will be proportional to the concentration of sodium ions in the blood. In the case of crabs living in 20–25 % sea water the concentration of sodium ions is roughly half the normal, and if the permeability of the fibre membrane does not change during the dilution of the blood, then the number of sodium ions entering the fibre in a given time will also be halved. When the new steady state has been reached the rate of secretion of sodium ions from the fibre must also be halved, and since the work done in transporting 1 gm.-ion will still be the same (since neither the membrane potential nor the Na_o/Na_i ratio is altered), then the total secretory work in pumping out sodium ions must also be halved. Thus the Na-pump mechanism is regulatory in nature, since its activity varies with the blood sodium concentration and serves to maintain a constant Na_o/Na_i ratio.

Another important feature of the sodium concentration in the muscle is that it closely approximates to the chloride concentration. The scatter diagram for these two variables is shown in Fig. 6 and the correlation coefficient $= 0{\cdot}72$; $P \ll 1\,\%$. The exact significance of this striking correlation is not known—it may simply reflect the close correlation between the blood sodium and chloride and the constancy of the Na_o/Na_i and Cl_o/Cl_i ratios or it may indicate some more intimate linkage between the concentration of the sodium and chloride ions within the muscle fibres.

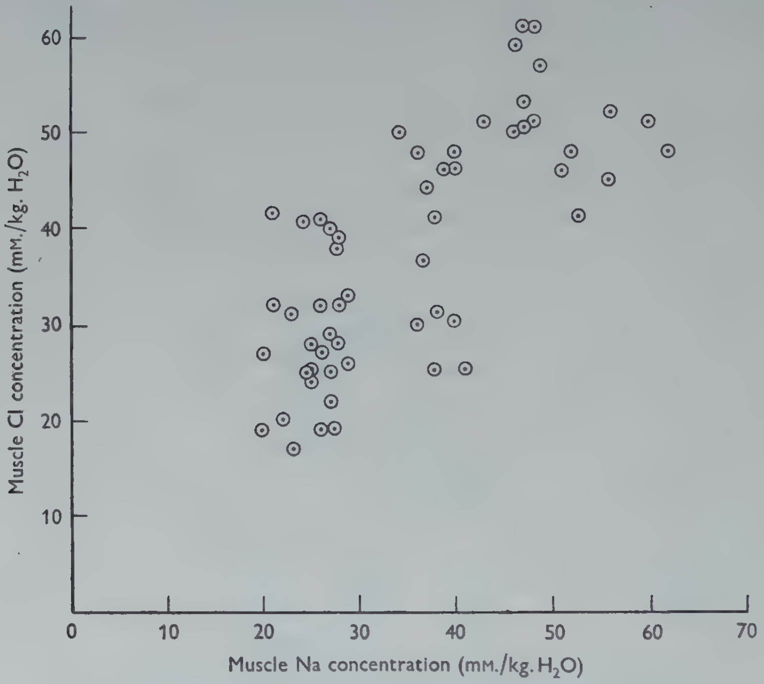


Fig. 6. The relation between the muscle sodium concentration and the muscle chloride concentration.

(c) *Potassium*

The concentration of potassium in the normal fibres is 120 mM./kg. water, K_o/K_i is 0·1 and the equilibrium potential for the potassium ion (+58 mV.) corresponds closely to the measured resting potential (Shaw, 1955). The relation between the potassium concentration in the diluted blood and the potassium concentration in the muscle is shown in Fig. 7. The muscle K falls roughly in proportion to the blood K until the latter reaches a value of about 8 mM./l.; at lower concentrations the drop in muscle K is not nearly so marked, and it tends to remain constant at about 80 mM./kg. water. This observation is more clearly demonstrated in Fig. 8 where the ratio K_i/K_o is plotted against the corresponding blood potassium concentration. Below 8 mM./l. this ratio steadily increases until at blood concentrations of about 4 mM./l. the ratio has approximately doubled. The extent to which these results can be taken to indicate the permeability of the fibre membrane to potassium will be discussed later. The increase in the K_i/K_o ratio which occurs at low blood potassium concentrations is not apparently accompanied by a corresponding increase in the membrane potential since, as we have seen, this remains approximately constant. In a few cases measurements of muscle potassium concentration and membrane potential were made on the same crab (taken from 20 % sea water) and this divergence was confirmed. If the permeability of the membrane to potassium is assumed, then these results would suggest that a mechanism capable of actively absorbing potassium from the blood might come into operation when the blood potassium concentration is sufficiently reduced.

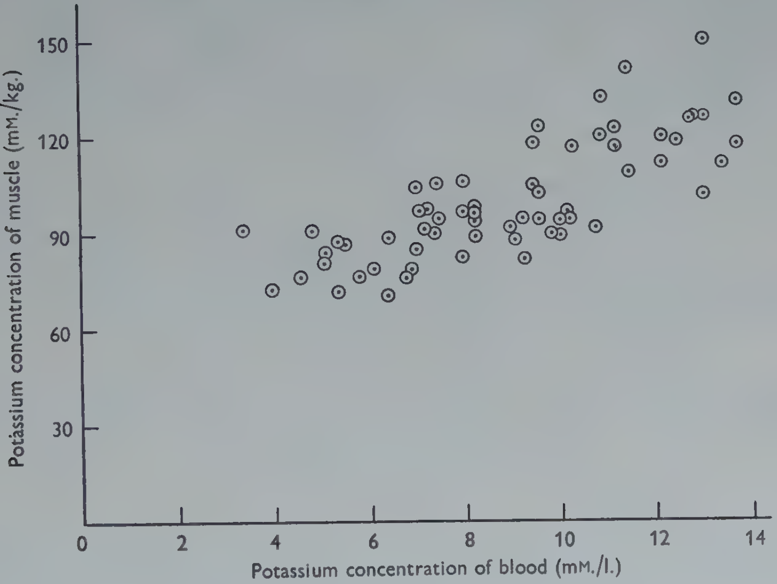


Fig. 7. The relation between the muscle potassium concentration and the blood potassium concentration.

(*d*) *Calcium and magnesium*

It is convenient to deal with calcium and magnesium together, since the behaviour of these two substances is very similar. The changes in concentration of these in the muscles as a result of blood dilution are not very great and therefore only the muscles from animals kept in 20–25 % sea water have been studied in detail. The results of the analyses of blood and muscle for both calcium and magnesium are given in Table 1. The blood calcium and magnesium are both reduced to a level

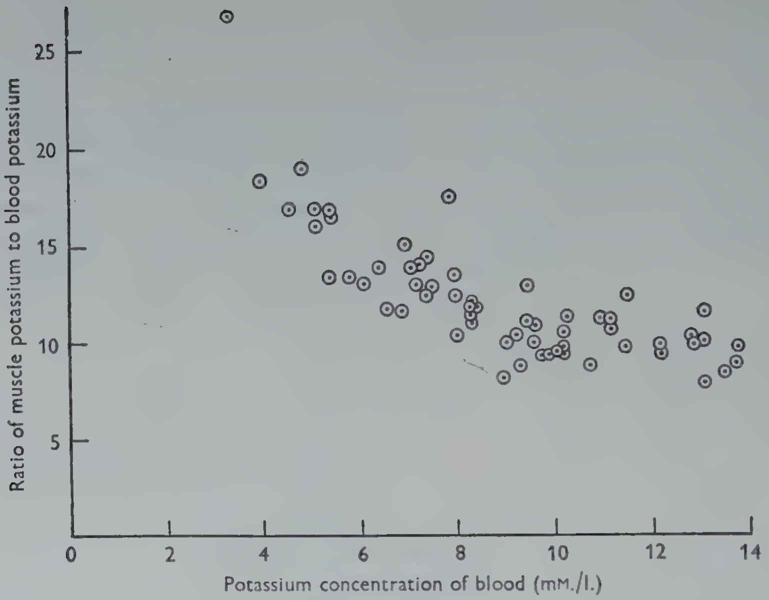


Fig. 8. The ratio muscle potassium concentration/blood potassium concentration as a function of the blood potassium concentration.

Table 1. *Concentrations of calcium and magnesium in blood and muscle of crabs from normal and dilute sea water*

(mM./kg. water)

Calcium				Magnesium			
Normal sea water		20–25 % sea water		Normal sea water		20–25 % sea water	
Blood	Muscle	Blood	Muscle	Blood	Muscle	Blood	Muscle
27·0	4·5	10·0	1·5	25·3	18·6	6·6	11·2
25·0	4·1	10·0	2·3	24·2	13·1	6·0	10·3
28·8	3·6	11·3	1·4	34·2	13·3	13·3	13·8
21·9	4·6	9·4	2·8	15·7	17·7	10·5	12·8
25·6	6·3	8·7	1·8	21·4	18·3	7·4	9·1
13·8	5·4	9·7	6·0	22·2	23·0	7·8	10·1
21·2	3·3	12·2	3·0	15·6	16·7	8·3	14·8
18·7	3·5	9·4	2·9	23·3	18·1	8·3	12·7
17·8	5·8	10·6	3·7	21·5	13·9	5·3	9·6
22·5	6·3	12·5	3·6	10·8	13·9	6·0	11·1
21·9	2·6	10·6	2·8	22·6	12·8	—	9·5
27·2	3·3	11·6	3·2	24·0	12·9	7·4	9·8
30·0	11·6	8·4	5·1	19·6	16·9	—	—
24·4	8·3	8·4	3·4	18·2	18·6	—	—
23·1	4·3	10·3	1·9	19·1	15·3	—	—
27·2	6·9	8·1	1·7	—	—	—	—
Mean 23·5	5·3	10·1	3·0	21·2	16·2	7·9	11·2
S.D. —	±2·3	—	±1·3	—	±2·9	—	±1·9

well below half that of normal blood, but the concentrations in the muscle do not fall to the same extent. The mean values for calcium and magnesium in the muscles of normal crabs, given in Table 1, agree with the values found by the analysis of single fibres (Shaw, 1955) and they are significantly higher than the concentrations found in the muscles of the crabs from the diluted sea water (for Ca, $t=3{\cdot}4$, $P<1\%$; for Mg, $t=5{\cdot}1$, $P<1\%$). These differences may mean that a proportion of the total calcium and magnesium has been lost from the fibres or they may be due to dilution as a result of the intake of water into the fibres. Now, allowing for the maximum swelling of the fibres the concentration of any fibre constituent would be reduced by about 20 %, and although the differences between the means for both calcium and magnesium suggest a somewhat larger fall than this, it would be difficult to decide from the data given in Table 1 if a significant drop had still occurred after due allowance had been made for the water increase in each case. For this reason a series of critical experiments was performed in which the blood calcium and magnesium were reduced but no swelling of the fibres took place. This was accomplished by keeping a batch of crabs in a 20 % sea-water solution to which sufficient NaCl had been added to make it isotonic with normal sea water and enough KCl to bring the potassium concentration to normal. Crabs would survive in this solution for at least 2 weeks. Analyses for calcium and magnesium in blood and muscle were made after 7–10 days and the results are given in Table 2. The mean water content of the muscles after this period was 73·6 % (S.D. ± 1·2 %) and this was not significantly different from the water content of normal muscles—thus no swelling had occurred. The mean values for the concentrations of calcium and magnesium in the muscles of these experimental animals did not differ significantly from the similar values for normal crabs (from Tables 1 and 2. For Ca, $t=1{\cdot}2$, $P>5\%$; for Mg, $t=0{\cdot}83$, $P>5\%$). There is, therefore, clearly no loss of calcium or magnesium from the fibres despite the substantial reduction in the concentrations of calcium and magnesium in the blood, at least during this period of time, and the fall

Table 2. *The concentrations of calcium and magnesium in the blood and muscles of crabs from 20 % sea water made isotonic by the addition of* NaCl

(mM./kg. water.)

Calcium		Magnesium	
Blood	Muscle	Blood	Muscle
6·9	5·6	18·0	14·3
6·9	2·1	7·1	13·8
6·2	3·3	8·1	15·1
7·3	5·1	10·4	14·0
7·3	4·3	12·2	17·5
6·7	3·1	9·4	16·8
13·1	5·2	11·3	13·1
—	—	10·6	15·7
—	—	10·4	17·8
Mean 8·7	4·1	10·8	15·3
S.D. —	±1·3	—	±1·7

in concentration found in the muscles of the crabs from the dilute sea water is most probably simply a result of swelling of the fibres and consequent dilution of their contents.

Two important deductions follow from these results. In the first place the simplest interpretation of the data is that the fibre membrane is impermeable to calcium and magnesium, and secondly the fall in the concentration of these ions is large enough to make it very probable that the fibres swell to the full calculated value.

DISCUSSION

In order to build up an integrated picture of the new equilibria which are set up in the muscles as a result of dilution of the blood, it is simplest to deal with sodium and chloride together and separate from the other inorganic ions. It has been demonstrated that the membrane of the fibres is permeable to these ions and that during the establishment of the new steady state with the diluted blood a proportion of them is lost from the fibre so that their final concentrations are about one-tenth of those in the blood. Although the loss of the chloride could be accounted for by a simple diffusion process, the sodium ions must be removed by a secretion process. The regulatory nature of this secretory activity has been pointed out, and the simplest theory that can be advanced to explain the regulation of the concentration of sodium ions within the muscle fibre is that the rate of transport of these ions out of the fibre is proportional to the internal concentration of sodium.

Table 3. *The electrolyte composition of muscles of crabs from normal and from 20 % sea water*

(Average values in m. equiv./kg. water.)

	Normal sea water	20 % sea water
Potassium	120	85
Sodium	50	29
Calcium	10·6	6
Magnesium	32·4	22·4
Sum of cations	213	142·4
Chloride	50	21
Unknown anions (by difference)	163	121·4

In order to interpret the behaviour of the remaining inorganic ions, further information is required of the total concentration of ions in the fibre. No direct measurements have been made, but an estimate can be formed assuming that sodium, potassium, calcium and magnesium are the only cations present, and that to preserve electro-neutrality these are balanced by an equal number of anions. This calculation does not take into account the fact that some of the cation fraction may not be ionized, but the proportion of un-ionized cations in the normal fibre is probably quite small (Shaw, 1955). Table 3 shows the mean concentrations of cations in the muscles of animals from normal sea water and from 20 % sea water. In the former, the total cation concentration is 213 m. equiv./kg. water and this falls to 142 m. equiv./kg. water—a fall of exactly one-third. One can conclude from this that the maintenance of the total ionic strength is not necessary for the normal functioning of the muscle cell. If these totals also represent the total anion concentrations, then an estimate of the remainder of the anion fraction can be arrived at by the difference between these and the measured concentrations of chloride. The differences are also shown in Table 3, where it is seen that the unknown anion fraction falls from 163 m. equiv./kg. water to 121. Little is known of the chemical nature of this fraction except that dicarboxylic amino-acids are practically absent (Lewis, 1952), but in vertebrate muscle cells (Boyle & Conway, 1941) it is supposed that this fraction consists largely of protein and large organic anions to which the fibre membrane is impermeable. In the case of the crab muscle fibre, if we make due allowance for the swelling which occurs in the fibres in diluted blood, then the expected fall in concentration of this anion fraction would be 20% of 163 and the final concentration 130 m. equiv./kg. water. Comparison of this figure with the value derived in Table 3 (121) shows that although there may have been a small leakage of anions (possibly of some of the smaller anions such as bicarbonate) the greater part of the fall can be accounted for by the swelling of the fibres. The simplest interpretation of these facts is that the fibre membrane is not permeable to the larger part of this unknown anion fraction.

The mechanism of the retention of potassium in the fibre can now be discussed since it is this ion which largely balances the non-chloride anion fraction. It has been shown that the potassium concentration in the fibres falls roughly in proportion to the blood potassium as the blood is diluted until a value of between 80 and 90 mM./kg. has been reached, and thereafter remains practically constant. To interpret these changes the effect of the swelling of the fibres in reducing the intracellular concentration of potassium must be calculated. This has been done for a large number of individual cases using the average concentration of potassium in normal fibres (120 mM./kg. water) and the average water content (74·5%) and computing the expected potassium content of the muscle (from dilute sea water) from the measured water content of that muscle. These calculations are given in Table 4, together with the measured potassium concentration. The correspondence between the calculated and measured values is in many cases quite close except for the crabs from 20% sea water where the divergence is greater. Thus the fall in concentration of potassium in the muscle fibres can be accounted for largely by the increase in water content of the fibres due to swelling, and again the simplest explanation would be that the fibre membrane is also impermeable to potassium. There are, however, a number of reasons for believing that this is unlikely to be the case. In the first place the fall in potassium concentration of the fibres of crabs from 20% sea water is rather greater than can be explained simply by swelling (Table 4), and preliminary experiments on crabs kept in normal sea water with a reduced potassium content have shown a significant fall in the potassium concentration of the muscles, although the fibres have not changed in volume. Secondly, important evidence comes from a study of the electrical properties of crab muscle fibres carried out by Fatt & Katz (1953). These authors found that the transverse resistance of the muscle fibre membrane was very low (the mean value for *Carcinus* fibres was 165 Ω-cm.^{2}) and considerably smaller than, for example, the frog muscle fibre membrane where the resistance is between 2000 and 4000 Ω-cm.^{2} (Fatt & Katz, 1951; Castillo & Machne, 1953). These estimates of membrane resistance are made from a knowledge of the current that flows through the membrane when the resting potential is increased by about 20 mV. above normal, and it does not necessarily follow that this current is carried by potassium ions; but, on the other hand, Fatt & Katz (1953) also showed that the membrane potential could be immediately reduced by the application of potassium-rich Ringer to the outside of the muscle fibres so the potassium seems to be involved at least in part. The permeability of the membrane to potassium is also suggested by the fact that it has been established by the use of radioactive potassium and by other means that many cells including vertebrate muscle cells exchange potassium rapidly with the external solution (Keynes, 1954, and see reviews by Krogh, 1946, and Hodgkin, 1951).

Table 4. *A comparison between the measured potassium content of muscles of crabs from dilute sea waters and the concentration calculated from their water content*

20 % sea water		30–40 % sea water		50–70 % sea water		85 % sea water	
K calc.	K found	K calc.	K found	K calc.	K found	K calc.	K found
93	79	120	98	97	95	115	119
105	105	104	95	101	95	117	124
116	106	101	90	103	98	120	121
82	77	101	85	96	91	125	118
76	74	92	92	101	98	113	105
93	85	103	88	99	90	109	107
94	73	90	82	95	97	—	—
103	88	94	90	109	106	—	—
97	71	90	94	102	103	—	—
95	91	94	97	97	88	—	—
91	71	107	97	97	89	—	—
94	92	90	82	90	94	—	—
Mean 95	84	99	91	99	95	117	115

If the membrane of the *Carcinus* muscle fibre is permeable to potassium then the retention of potassium within the fibre when the blood potassium concentration is below 8 mM./l. must be brought about by a specific transporting mechanism capable of absorbing potassium ions from the blood. The presence in cells of active transporting mechanisms which are specific for potassium ions is by no means unknown. The presence of such a process has been described, for example, in vertebrate red blood cells (Maizels, 1954), and recently it has become clear that the influx of potassium ions into cephalopod giant axons (Hodgkin & Keynes, 1954) and, possibly, also into frog muscle fibres (Keynes, 1954) is not a simple diffusion process but seems to be linked to the sodium-transporting mechanism.

A simple theory that could account for the behaviour of the potassium ions in *Carcinus* muscle is that the influx of potassium is always mediated by a transport system, although in the normal fibre no metabolic energy would be required to drive it. If the rate of transport of potassium ions, inwards, was proportional to the internal potassium concentration rather than to the potassium concentration in the blood, then the retention of potassium in the muscle would be a consequence. Perhaps the energy which can be diverted to this system is limited and it slows down a little at very low blood potassium concentrations.

We are now in a position to see to what extent the theory of Boyle & Conway is applicable to the muscles of *Carcinus* in diluted blood. The two requirements of the theory are that the membrane shall be impermeable to the bulk of the anion fraction, to sodium and to large ions such as calcium and magnesium, but permeable to small ions such as potassium and chloride and that as a result of the Donnan equilibrium

$$\mathrm{Cl}_o/\mathrm{Cl}_i = \mathrm{K}_i/\mathrm{K}_o = \exp\,(nFE/RT),$$

where E is the membrane potential.

All the requirements for the permeability of the membrane are fulfilled except in regard to sodium. The theory will not explain the distribution of this ion, but this does not invalidate the theory completely since the sodium concentration in the muscle is always kept low. The predicted relationships for chloride and potassium are obeyed well in crabs taken from dilutions of sea water down to 40%—it is only at the very high dilutions that the divergence in the potassium concentration becomes apparent. The reason for the retention of potassium ions and the removal of sodium ions from the fibres during the setting up of a new steady state remains obscure. The maintenance of a high potassium concentration in cells is so widespread that it appears that it must be necessary for the normal functioning of protoplasm, although this has never been clearly demonstrated. In the crab muscle fibres the actual concentrations of sodium and chloride ions do not seem to be very critical, since the fibres can tolerate a wide range of concentrations and, of course, in vertebrate muscle cells the concentration of these ions is very low. It seems as though the sodium-ion concentration is regulated to neutralize the charge due to the presence of chloride ions in the fibre and the potassium-ion concentration regulated to balance the charge of the impermeable anion fraction, the concentration of the anions being determined solely by the degree of swelling of the fibres and the membrane potential.

If this interpretation is correct, then the extent to which these muscle cells can withstand and adapt themselves to a dilution of the blood will depend on their ability to control water intake and resist swelling during the re-establishment of a steady state.

SUMMARY

1. The electrolyte composition of the carpopodite extensor and flexor muscles of the chelae of *Carcinus maenas* living in diluted sea waters has been studied.
2. The increase in water content of the muscles of crabs from 20% sea water corresponds to a maximum degree of swelling of 25% of the initial weight.
3. The resting membrane potential remains constant at 59 mV. over the whole range of blood concentrations studied.
4. Chloride is lost from the fibres and its concentration in the muscle is always about one-tenth of that in the blood.

5. Sodium ions are actively secreted from the muscles, and a steady state is reached when the concentration in the muscle is also about one-tenth that in the blood. The sodium and chloride concentrations in the muscle are approximately the same.

6. The potassium concentration of the muscle falls as the blood is diluted, but the ratio of muscle potassium to blood potassium increases from 10 to about 20. Potassium appears to be actively retained in the muscle.

7. The fall in concentration of muscle calcium and magnesium can be explained in terms of the swelling of the fibres alone and the fibre membrane is probably not permeable to these substances.

8. The fibre is also probably impermeable to the larger part of the anion fraction. It is only sodium and potassium ions which are actively regulated and the result of these processes is that the muscle sodium balances the muscle chloride and the muscle potassium balances the impermeable anion fraction. It is suggested that on the extent to which the muscle cell can withstand blood dilution depends the degree to which it can resist swelling.

This work was assisted by a grant from the Royal Society.

REFERENCES

BOYLE, P. J. & CONWAY, E. J. (1941). Potassium accumulation in muscle and associated changes. *J. Physiol.* **100**, 1.

CASTILLO, J. DEL & MACHNE, X. (1953). Effect of temperature on the passive electrical properties of the muscle fibre membrane. *J. Physiol.* **120**, 431.

DEAN, R. B. (1941). Theories of electrolyte equilibrium in muscle. *Biol. Symp.* **3**, 331.

FATT, P. & KATZ, B. (1951). An analysis of the end-plate potential recorded with an intracellular electrode. *J. Physiol.* **115**, 320.

FATT, P. & KATZ, B. (1953). The electrical properties of crustacean muscle fibres. *J. Physiol.* **120**, 171.

HARRIS, E. J. & BURN, G. P. (1949). The transfer of sodium and potassium between muscle and the surrounding medium. *Trans. Faraday Soc.* **45**, 508.

HODGKIN, A. L. (1951). The ionic basis of electrical activity in nerve and muscle. *Biol. Rev.* **26**, 339.

HODGKIN, A. L. & KEYNES, R. D. (1954). Movements of cations during recovery in nerve. *Symp. Soc. Exp. Biol.* **8**, 423.

KEYNES, R. D. (1954). The ionic fluxes in frog muscle. *Proc. Roy. Soc.* B, **142**, 359.

KROGH, A. (1939). *Osmotic Regulation of Aquatic Animals.* Cambridge University Press.

KROGH, A. (1946). The active and passive exchanges of inorganic ions through the surfaces of living cells and through living membranes generally. *Proc. Roy. Soc.* B, **133**, 140.

LEVI, H. & USSING, H. H. (1948). The exchange of sodium and chloride ions across the fibre membrane of the isolated frog sartorius. *Acta physiol. scand.* **16**, 232.

LEWIS, P. R. (1952). The free amino-acids of invertebrate nerve. *Biochem. J.* **52**, 330.

MAIZELS, M. (1954). Active cation transport in erythrocytes. *Symp. Soc. Exp. Biol.* **8**, 202.

SHAW, J. (1955). Ionic regulation in the muscle fibres of *Carcinus maenas*. I. The electrolyte composition of single fibres. *J. Exp. Biol.* **32**, 383.

STEINBACH, H. B. (1951). Sodium extrusion from isolated frog muscle. *Amer. J. Physiol.* **167**, 284.

WEBB, D. A. (1940). Ionic regulation in *Carcinus maenas*. *Proc. Roy. Soc.* B, **129**, 107.

FURTHER STUDIES OF GAS-FILLING IN THE INSECT TRACHEAL SYSTEM

By JOHN BUCK AND MARGARET KEISTER
National Institutes of Health, Bethesda 14, Maryland, U.S.A.

(*Received* 18 *February* 1955)

I. INTRODUCTION

During the moulting of the larvae of many insects the gas-filled tracheae of one stage are shed with the cast-off external cuticle. The new and larger tracheal system of the succeeding stage at first contains only liquid, but normally it soon fills with gas. Although in some species the gas is atmospheric air entering through the spiracles, there are many in which the gas first appears within the tracheal system, with no contact with an outside source.

The *de novo* type of gas-filling, in particular, has stimulated much work and speculation (for reviews see Keilin, 1924; Sikes & Wigglesworth, 1931; Keister & Buck, 1949*a*; Wigglesworth, 1953). Although earlier workers had postulated an 'absorption' of the tracheal liquid in conjunction with various sources of tracheal gas, Keilin was the first to propose clearly that 'a sudden imbibition...or chemical reaction taking place in the cells' of the peritracheal epithelium could, by breaking the column of intratracheal liquid, lead to the simultaneous disappearance of the liquid and appearance of the gas.* Keilin's 'chemical reaction' obviously refers to the sort of mechanism which would commonly be called to-day—and will be so called by us—'active uptake' or 'metabolic uptake'. This process implies a net transfer of liquid in circumstances requiring expenditure of energy by the cell (i.e. with decrease in entropy, thermodynamically speaking). It is premature to speculate about a specific transfer mechanism, but presumably it could be either aerobic or anaerobic, and could involve either active uptake of water itself or active uptake of solute followed by osmotic transfer of water.

Keilin's hypothesis has been developed particularly by Wigglesworth. By assuming that tracheal gas-filling in insects which need contact with atmospheric air differs from that in insects able to fill from an internal source only in that the uptake forces are not strong enough to rupture the liquid column directly, he has been able to knit together data from a wide variety of material. Using mosquito larvae he showed (1938) that the gas-filling could be inhibited by chloroform anaesthesia, the first direct evidence that an active mechanism ('secretion') might be involved. Using larvae of the mycetophilid fly *Sciara*, we obtained additional evidence on this point by showing that the disappearance of the tracheal liquid can be delayed by cold, and found further that the process is an obligatorily aerobic one, and that the gas-filling is independent of the nature of the gas with which the tissues are saturated, provided that a minimal concentration of oxygen is present (Keister & Buck, 1949*a*). In this work we observed that oxygen concentrations of 0·3–0·5% immobilized the freshly moulted larvae but did not interfere with gas-filling. The fact that the filling begins suddenly just after moulting therefore suggests that exposure of premoult larvae to low oxygen might halt the body movements necessary for moulting and indicate whether the morphological changes which are normally visible at that time are prerequisites for the filling.

---

* Although this is a logical and attractive proposal, changes in tracheal gas content are not necessarily linked to movements of liquid. Herford (1938), for example, has shown that flea tracheae may alternately inflate with gas and deflate, due, probably, to respiration, and in the *Phormia* larva Buck & Keister (1956) have demonstrated both tracheal collapse and inflation caused purely by gas diffusion.

In order to clarify the plan of investigation we summarize the following results, mainly from our previous reports (Keister & Buck, 1949*a*, *b*): (*a*) the tracheae which are to serve the fourth-stage larva are fully formed in the mature third-stage larva before moulting and are liquid-filled. (*b*) The onset of moulting is signalled by definite and readily recognized changes in larval behaviour which may last for some minutes. This premoult behaviour involves periods of axial back-and-forth rotation of the proventriculus and of slow strong peristalses of the body wall, alternately forward and backward, and culminates in a repeated bulging of the body just behind the head capsule which eventually splits both capsule and cuticle longitudinally, permitting the new fourth-stage larva to crawl forward out of the anchored old skin. The duration of the process is quite variable, but ordinarily not more than 15 min. elapse between the first recognizable behaviour and moulting itself. Both the behaviour and moulting are inhibited by total anoxia and by cold. (*c*) In normal (postmoult) gas-filling, there is a sharp distinction between total anoxia, in which filling is inhibited, and O_2 concentrations from 0·3 to 100%, in which normal filling occurs. (*d*) Mature *pre*moult larvae never gas-fill in air—i.e. air is normally associated with *liquid*-filled fourth-stage tracheae before the third moult (i.e. while the larva is still in the third stage) and with *gas*-filled fourth-stage tracheae after the moult. (*e*) In cultures which have been refrigerated for several days an occasional larva is found which has shed its skin and old tracheae, and in which the new tracheal system has filled with gas, but which still has an intact third-stage head capsule. Although such occurrences are abnormal they indicate that the actual splitting and shedding of the head capsule, events which are invariable forerunners of *normal* filling, are not prerequisite for the appearance of gas in the tracheae.

II. MATERIAL AND METHODS

Larvae of *Sciara coprophila* Lintner were raised in Petri dish cultures. Late third-stage larvae were used individually as soon as recognizably in one of the stages of premoult behaviour previously described. Response to gases was tested in cylindrical gas chambers (Fig. 1), two to four usually being run in tandem by two observers. Flushing rate was gauged by a water bubbler on the inlet side, which also aided the wet filter-paper lining of the chamber walls in keeping the humidity high. The larvae were held against the under side of the chamber cover-glass by a thin water film. The gases used were 0·5, 0·75 and 1·0% O_2 in N_2, and commercial prepurified N_2 (<0·1% O_2), water pumped N_2 (0·3% O_2), He (0·00% O_2) and CO_2 (0·00% O_2).

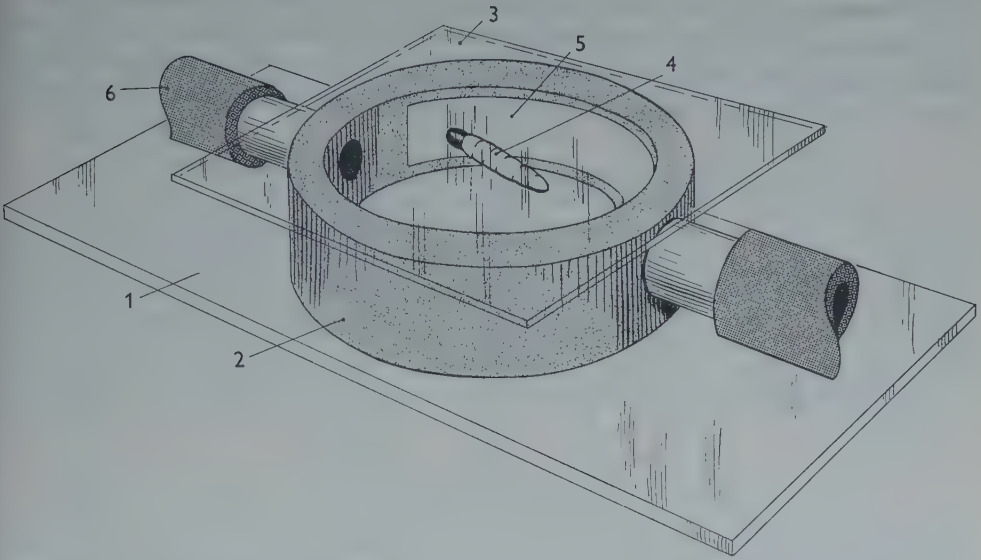


Fig. 1. Chamber for exposure of *Sciara* larvae to gases. 1, Glass microslide; 2, brass cylinder, $\frac{7}{8}$ by $\frac{5}{16}$ in., cemented to slide with de Khotinsky cement; 3, cover-glass sealed on with vaseline; 4, larva; 5, moist filter-paper lining (several layers); 6, inlet tube.

III. RESULTS

Third-stage larvae which were exhibiting premoult behaviour were exposed to various gas mixtures. The results are summarized in Table 1, in which we shall first consider only the data given in the first four columns (to the left of the heavy line).

A. *Effects of hypoxia on premoult larvae*

When premoult larvae were exposed to 0·3–0·75% O_2 in N_2, concentrations which permit tracheal gas-filling in freshly moulted larvae but which prevent body movement, the tracheal system gas-filled at least partially in a high proportion (33–100%) of individuals (lines 1–3, Table 1), the gas appearing first in the large trunks. Of those which did not fill on first exposure, a further high proportion filled if 'primed' by being returned to air for a brief period and then re-exposed to low O_2 (lines 5 and 6). These tests show at once that moulting *per se* is not a prerequisite for the appearance of gas in the fourth-stage tracheae.

Even if the actual shedding of the third-stage skin and tracheae is not necessary for gas-filling it is possible that some preliminary to moulting effects a mechanical change essential for filling, and indeed this possibility cannot be excluded since premoult larvae were selected only on the basis of characteristic movements. For example, it could well be imagined that the peristalsis and rotating stages of premoult behaviour might draw out the third-stage tracheae from the liquid-filled fourth-stage tracheae, even though the skin itself remained intact. The possibility of the third-stage tracheae being 'loosened' cannot be excluded, but direct microscopic examination shows clearly that the air-filled third-stage tracheae are still *in situ* in the fourth-stage trunks as the latter fill with gas. Furthermore, in the minority of premoult larvae that do not gas-fill in low O_2 it frequently happens that there is a gradual accumulation of liquid between the third- and fourth-stage cuticles, perhaps by osmotic uptake of water through the third-stage cuticle into the moulting fluid. In such instances the outer skin stretches, the intersegmental folds straighten out and the larva eventually appears enclosed in a transparent sac which is attached only at the neck just behind the head capsule, and sometimes at the proctodaeum, and is widely separated from the fourth-stage skin. This separation of the third-stage skin of course does at least partially draw out the third-stage tracheae, but does not induce gas-filling of the fourth-stage tracheae.

Table 1. *Gas-filling and reversal of filling in premoult larvae*

Gas	No. larvae tested	No. larvae gas-filling (without moulting)	% gas-filled	No. larvae reversed (liquid-filled) (still not moulted)	% reversed
		On 1st exposure			
Water pumped tank N_2	37	16	43	15 (15 tested)	100
0·5 % O_2 in N_2	9	3	33	3	100
0·75 % O_2 in N_2	6	6	100	All moulted	—
1 % O_2 in N_2	17	1	6	1	(100)
		After air-primes			
Water pumped tank N_2 after air-primes	8	7	88	7	100
0·5 % O_2 after air-primes	6	4	66	3	75
0·3, 0·75 or 1 % O_2 after various gases	63	32	51	26	81
		On 1st exposure			
Prepurified or furnaced N_2	70	18	26	12 (13 tested)	92
He	34	7	20	5 (6 tested)	83
CO_2*	8	1	12·5	0	0
Totals	181†	95	—	72	—

* CO_2 of course had a narcotic as well as anoxic effect.

† Total number tried does not include the seventy-seven tried after priming or other gases because they are already counted in the other groups, i.e. these are the 'failures' in direct filling tests.

Premoult larvae in which the tracheae did not fill with gas in low O_2, even after being primed (lines 5–7, Table 1, amounting to about 35 % of all larvae tested in low O_2), could, on return to air, either: (*a*) recover, moult and remain liquid-filled; (*b*) recover, moult and fill with gas, as normally occurs; (*c*) recover temporarily, but neither moult nor gas-fill; (*d*) recover, gas-fill and then partially moult; or (*e*) recover, gas-fill, but fail to moult. Consequence (*a*) is definitely abnormal, although survival is not surprising in view of the fact that larvae can complete development even with kerosene-filled tracheal systems.* Consequence (*b*) requires no explanation. Consequence (*c*) indicates injury, or possibly a change of the type seen in *Aedes* larvae, which, with increasing time of forced submergence, rapidly lose their capacity to gas-fill (Wigglesworth, 1938). Consequences (*d*) and (*e*), though undoubtedly abnormal in the light of the usual effect of air on premoult animals (see below), at least fortify the main finding that gas-filling can occur without moulting.

B. *Effect of* 1 % O_2 *on premoult larvae*

Since we know that gas-filling of premoult larval tracheae never normally occurs in air, and since we have shown that it can occur in 0·3–0·75 % O_2, it is obvious that there must be some concentration greater than 0·75 % at which O_2 begins to inhibit gas filling. We have not investigated this point exhaustively, but, as shown in line 4 of Table 1, it appears that a concentration of 1 % O_2, which is too high to completely inhibit body movement, permits very little gas-filling prior to moulting.

C. *Effects of anoxia on premoult larvae*

Third-stage larvae exhibiting premoulting behaviour were exposed to helium (as an anoxic standard) and to nitrogen which was either commercially 'prepurified' or passed over hot copper gauze. These treatments caused an almost immediate cessation of body movement and of visible activity of internal organs such as heart and gut. While the larva was anoxic there was, in 77 % of the tests, no change in the liquid-filled fourth-stage tracheae (lines 8–10, Table 1). The results were the same whether the larva was apparently in the early stages of rotating the proventriculus or in the bulging stage immediately before the splitting of the head capsule. Larvae were able to recover and moult after at least 1½ hr. of anoxia, showing the possibility of postponing moulting and postmoult gas-filling far beyond the normal time. Premoult larvae thus resemble freshly moulted larvae in that their gas-filling is dependent on O_2.

The twenty-six exceptional instances of gas-filling which occurred in ostensibly anaerobic conditions were conspicuously slower and less complete than in premoult larvae gas-filling in low O_2 concentrations, and seem quite reasonably explained as due to failure to flush out the gas chambers sufficiently rapidly and completely. The flushing presents a particularly difficult problem, first, because the dilution of the O_2 in the chamber by the anoxic gas proceeds proportionately more slowly the lower the pO_2 becomes, and secondly, because in order to become anoxic the larva must pass through the critical region of O_2 concentration—0·3–0·75 %—which induces or permits gas-filling.

---

* Such larvae pupate apparently normally but do not complete adult development. The fact that they can live as fourth-stage larvae for 5 or 6 days restricted to cutaneous respiration indicates that the tracheal system may not be important in normal respiration. However, the tracheal system is not of the closed type but has the normal dipteran complement of open spiracles, as shown by Keister (1948) and Keister & Buck (1949*a*). The fact that gas appears first in the interior of the tracheal system, therefore, is not because entry of air through the spiracles is impossible.

At least fifty-one of the eighty-six larvae which did not gas-fill in anoxic gases did later fill under various other conditions, most of them prior to moulting, indicating that the anoxia was not in itself injurious.

D. *Effect of air on gas-filled premoult larvae*

If gas-filled or partially gas-filled premoult larvae, obtained as outlined in section A above, were put back in air a most unexpected thing occurred. In more than 75 % of the tests (right side of Table 1) the tracheae refilled with liquid. This reversal of gas-filling is itself reversible, and in some larvae the tracheae could be filled again with gas (by subjecting the larva again to low pO_2) and then refilled with liquid (by re-exposure to air) five successive times before the larva finally moulted and filled its tracheae permanently with gas. These changes occurred without necessarily involving body movement.

The paradoxical nature of this reversal of gas-filling can perhaps best be appreciated by comparing the responses in moulted and premoult larvae. In both larvae the appearance of gas in the tracheal system can take place in O_2 concentrations of 0·3 to 0·75 %. In moulted larvae, however, higher concentrations such as in air accelerate, if anything, the replacement of tracheal liquid by gas, whereas in premoult larvae air causes the disappearance of the tracheal gas and its replacement by liquid.

Larvae which gas-filled or partly gas-filled in low pO_2, but failed to reverse in air, either moulted and then completed their tracheal gas-filling, or failed to moult and prematurely died.

E. *Effect of anoxia on partially gas-filled premoult larvae*

Premoult larvae which had started to gas-fill during exposure to low O_2 concentrations were exposed to presumably O_2-free N_2 (prepurified N_2 passed over hot copper gauze). In approximately 50 % of the tests gas-filling stopped immediately. In other instances, gas-filling continued slowly for various periods of time. The positive results can be interpreted as supporting the conclusion that appearance of gas in the tracheae is absolutely dependent on O_2 (section C above, and previous findings with moulted larvae). Instances of failure to halt completely the advance of gas in the tracheae, though conceivably reflecting an O_2-insensitive stage in gas-filling, are much more reasonably attributed to failure to attain absolute anoxia.

Larvae in which gas-filling was stopped by anoxia could either (*a*) resume filling upon re-exposure to low O_2 concentration, or, upon exposure to air, (*b*) reverse and become liquid-filled, (*c*) remain partially gas-filled, moult, and then complete gas-filling, (*d*) remain partially gas-filled without moulting.

F. *Relation of stage of moulting to tracheal gas-filling*

Regardless of whether the change which brings about the release of gas into the tracheae is physico-chemical or morphological, one would expect that it is a progressive change which normally reaches its spontaneously triggering level just after moulting. It seems reasonable, in other words, to anticipate that the 'ease' of inducing gas-filling of premoult larvae should increase with approach to moulting.

Admitting the possibility that we have incorrectly seriated the premoult behaviour stages, a tabulation of percentage 'success' (Table 2) does not support the expectation that conditions favouring gas-filling improve with time, and there is little indication that the frequency of reversal is greater the earlier the stage at which gas-filling occurs in intact premoult larvae. However (Fig. 2), there is some indication that reversal becomes less frequent as the dilapidation of the skin increases.

Table 2. *Frequency of gas-filling and of reversal in relation to stage of premoult behaviour at set-up time. Only first tests are included* (*no priming*)

Stage	No. larvae	No. filled	% filled	No. reversed	% reversed
Early	82	23	28	22	96
Middle	51	14	28	11	78
Late	48	8	17	6 (7 tested)	86

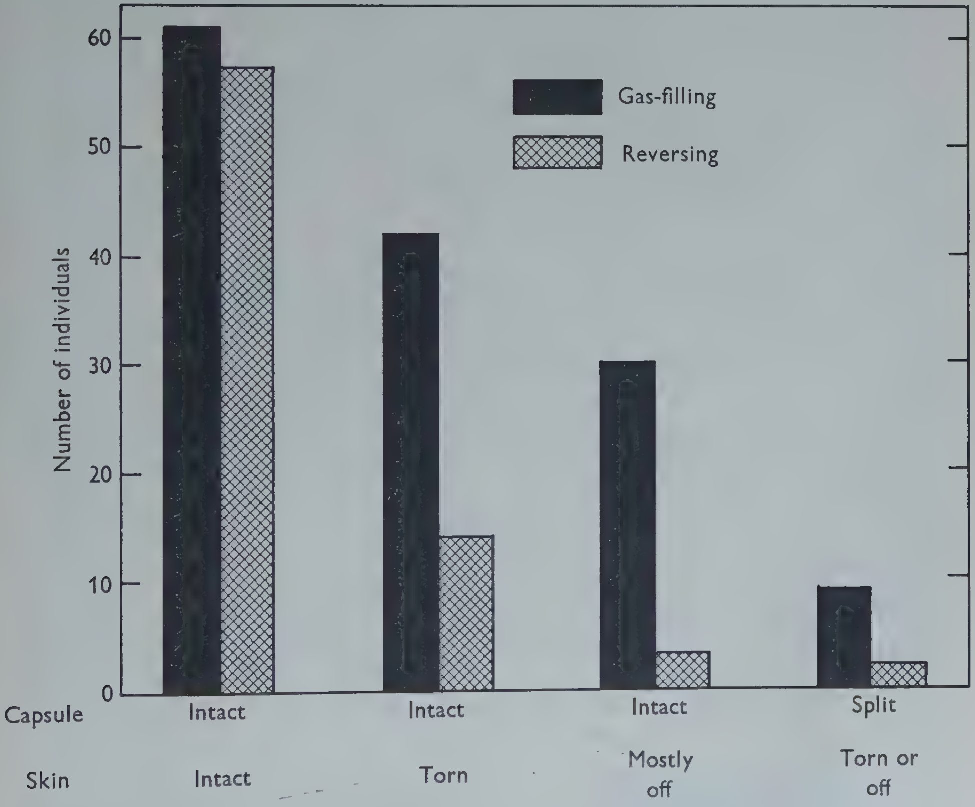


Fig. 2. Reversal of gas-filling in relation to condition of third-instar (premoult) head capsule and skin. The total number falls off with increasing dilapidation due to the high proportion which moult before completion of the experiments.

IV. DISCUSSION

It has been shown that by subjecting premoult third-stage larvae to low pO_2 it is possible to induce them to fill their new fourth-stage tracheae with gas without first moulting, as they normally would do. Since this filling will occur when cuticle and head capsule are intact, it seems clear that neither the splitting of the capsule nor moulting as a whole is the stimulus which sets off normal tracheal filling. The normal trigger for gas-filling must thus be sought in chemical changes in the tracheae or physiological alterations in contiguous tissues.

The gas-filling of premoult larvae resembles that of postmoult larvae—and is in so far compatible with the Keilin–Wigglesworth active uptake hypothesis—in being absolutely dependent upon oxygen. It also resembles almost all other known instances of gas-filling in beginning first in a large trunk and then spreading into smaller tracheae, presumably due to the fact that the liquid column in the largest tracheae has the smallest perimeter to cross-section ratio and hence is most easily broken.* However, it differs basically in its reversibility, and particularly in its response to air. Thus, paradoxically, air promotes premoult liquid-filling and postmoult air-filling (Fig. 3).

Wigglesworth (1930, 1938, 1953) has described a second liquid-gas exchange mechanism which is not related to moulting but applies particularly to the extension and retreat of gas in the tracheoles in response to variations in supply and demand of oxygen. This mechanism involves an equilibrium between capillarity, tending to withdraw liquid from the tracheal epithelium into the lumen, and the swelling pressure or imbibition of the cytoplasm tending to withdraw liquid from the tracheole. The equilibrium can be shifted by osmotic transfer of liquid from the tracheal epithelium into the blood due to an accumulation there of unoxidized metabolites. It thus differs from the ordinary postmoult active uptake mechanism in depending on physical, rather than on metabolic forces, and in being inhibited by increasing environmental oxygen concentration (which oxidizes the accumulated metabolites). Without considering the intrinsic merits of this hypothesis, which is supported by ingenious model experiments and persuasive observations, it is of interest to ascertain whether it offers a possibility of reconciling the premoult and postmoult gas-filling behaviour in *Sciara*. In this attempt we are happy to acknowledge the valuable suggestions of Prof. Wigglesworth.

If one postulates (*a*) that the concentration of metabolites accumulated in the blood is maximal in 0·3–0·75 % ambient oxygen, falling at lower oxygen concentrations (because there is no metabolism to form metabolites) and at higher (because the excess oxygen oxidizes the metabolites), and (*b*) that active uptake of liquid is weak or lacking in the premoult period, the following consequences might be expected:

---

* This deduction is not confined to active uptake mechanisms, nor can we conclude from it that the liquid is taken up through the walls of the large tracheae (though instances of gas-filling from two ends of an unbranched trunk show that this can occur: Wigglesworth, 1938; Keister & Buck, 1949*a*). A further point indicating physical influences on locus of first filling is our observation that filling often seemed to start at a tracheal fork. Keilin's fig. 2 suggests the same thing.

(1) In 0·3–0·75 % oxygen the larva gas-fills before moulting because of physical uptake of tracheal liquid due to accumulated metabolites (possibly augmented by weak active uptake).

(2) At oxygen concentrations greater than 1 % the tracheal system of the premoult larva refills with liquid (reverses) by capillarity because, the metabolites now being oxidized, the physical uptake mechanism is no longer operating and the active uptake mechanism has not developed or is still weak.

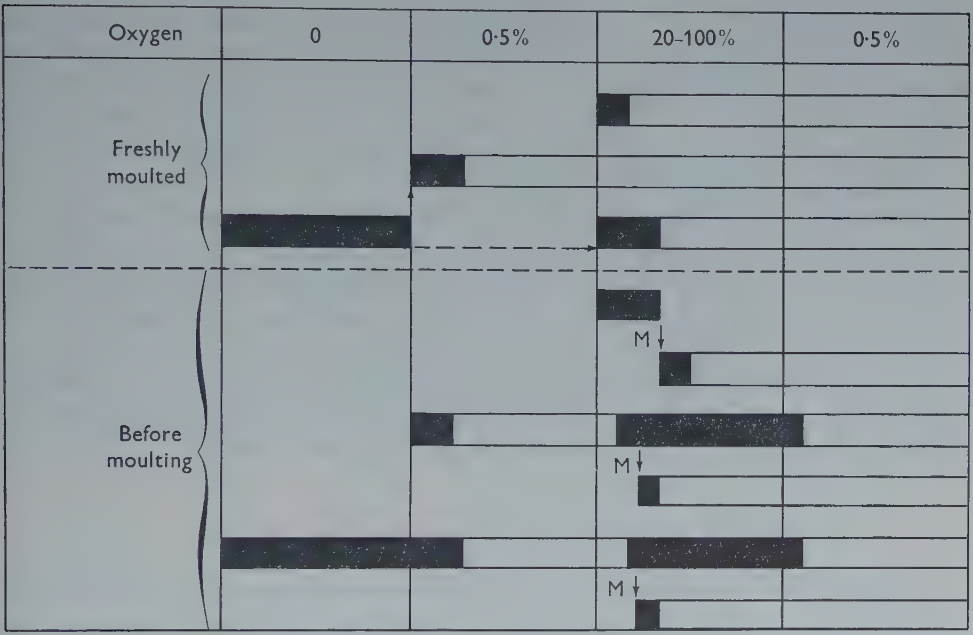


Fig. 3. Summary of gas-liquid phase changes in freshly moulted and premoult larvae in various O_2 concentrations. Solid black bars represent liquid-filled tracheae. Open bars represent gas-filled trachea. M indicates moulting. Four observed types of behaviour in freshly moulted larvae are illustrated above the dotted line; five in larvae just before the third moult are illustrated below.

(3) Air produces no reversal of gas-filling in postmoult larvae because in them the active uptake mechanism is stronger than the capillarity.

(4) Gas-filling of postmoult larvae is not prevented by 99·7 % CO_2 (Keister & Buck, 1949*a*) because the liquid can be withdrawn also by physical uptake (which is not subject to narcosis). (This lack of CO_2 inhibition was a previously unexplained exception to the active uptake hypothesis.)

The combination of Wigglesworth's active uptake and physical uptake mechanisms (plus appropriate postulates) thus provides an explanation of the two major characteristics by which premoult gas-filling differs from postmoult filling: its reversibility and its restriction to a narrow range of oxygen concentration. A number of discrepancies remain, although each could perhaps be reconciled with the major observations by additional not unreasonable assumptions. The first is that oxygen concentrations high enough to permit body movement of premoult larvae induce liquid-filling (reversal), whereas, according to Wigglesworth (1953) gas-filling 'is most active during asphyxia causing violent muscular activity in the absence of oxygen....'* A second is that anoxia does not bring about liquid-filling in either premoult or postmoult gas-filled larvae. Liquid-filling might be expected to occur because neither active nor physical liquid uptake is operating (since gas-filling will not occur in the absence of oxygen) yet capillarity remains. For the gas-filled postmoult larva the ineffectiveness of anoxia in inducing liquid-filling could perhaps be explained by the sort of postmoult tracheal tanning by which Wigglesworth explained the loss of ability to air-fill observed in mosquito larvae with increasing time of submersion in water, but this would not be compatible with reversibility in premoult filling. A third discrepancy with theory is the cold inhibition of postmoult gas-filling (Keister & Buck, 1949*a*), where one might have expected the physical uptake mechanism to continue.

In summary, it is possible to construct a partial explanation of both premoult and postmoult gas-filling in *Sciara* by postulating both metabolic and physical uptake of liquid. To the objection that the 'explanation' is little more than a redescription of the observations it can be said that there is considerable evidence for the existence of each mechanism separately, and that whatever the mechanism of gas-filling in *Sciara* may eventually turn out to be, it must explain the uniquely narrow range of oxygen concentration within which filling is possible in the premoult larva, and the opposite effects of air on premoult and postmoult larvae.

V. SUMMARY

1. In *Sciara* larvae exposed to total anoxia before moulting, all visible movement and all visible change in the content of the tracheal system cease. Moulting and tracheal gas-filling can be postponed at least 1½ hr. beyond normal time.
2. In most third-stage larvae exposed to 0·3–0·75 % O_2 before the third moult, the future fourth-stage tracheal system, which is present fully-formed in the body, fills with gas. This shows that although moulting invariably precedes gas-filling under normal circumstances it need not do so.
3. In premoult larvae which have filled their trachea with gas upon exposure to 0·3–0·75 % O_2, the tracheae fill again with liquid when the larvae are put back into atmospheric air. This reversal of gas-filling can be alternated with gas-filling several times in the same individual.

---

* A further indication of non-effect of muscular activity *per se* was obtained in putting a drop of ethyl ether on the cover-glass of the gas chamber so as to chill the cover and renew by condensation the water film around occasional larvae which had begun to become dry from long sojourn in flowing gas. The chilling caused the larvae to contract suddenly and extremely, after which they slowly re-elongated as the cover-glass warmed. Even repeated chilling, however, did not induce gas-filling in anoxic larvae. When larvae with partially gas-filled trunks were so chilled the tracheal gas first contracted (i.e. retreated), then expanded again to its original position.

4. The fact that in reversal of gas-filling an increase in pO_2 promotes liquid-filling, whereas in moulted larvae it not only never leads to liquid-filling but actually accelerates gas-filling, indicates that some basic, but at least temporarily reversible physiological or chemical change occurs in the tracheae or in the metabolism of the peritracheal tissue, near the time of moulting. A partial explanation of the observed phenomena can be made in terms of a combination of active uptake and physical uptake of tracheal liquid. Evidence for the existence of both types of mechanism, separately, has been adduced by Wigglesworth in other material.

VI. REFERENCES

BUCK, J. B. & KEISTER, M. L. (1956). Diffusion phenomena in the tracheae of the *Phormia* larva. *Physiol. Zoöl.* (in the Press).

HERFORD, G. M. (1938). Tracheal pulsation in the flea. *J. Exp. Biol.* **15**, 327–38.

KEILIN, D. (1924). On the appearance of gas in the tracheae of insects. *Proc. Camb. Phil. Soc. Biol. Sci.* **1**, 63–70.

KEISTER, M. L. (1948). The morphogenesis of the tracheal system of *Sciara*. *J. Morph.* **83**, 373–424.

KEISTER, M. L. & BUCK, J. B. (1949*a*). Tracheal filling in *Sciara* larvae. *Biol. Bull., Woods Hole*, **97**, 323–30.

KEISTER, M. L. & BUCK, J. B. (1949*b*). Reversibility of tracheal filling in *Sciara* larvae. *Anat. Rec.* **105** (3), 5.

SIKES, E. K. & WIGGLESWORTH, V. B. (1931). The hatching of insects from the egg and the appearance of air in the tracheal system. *Quart. J. Micr. Sci.* **74**, 165–92.

WIGGLESWORTH, V. B. (1930). A theory of tracheal respiration in insects. *Proc. Roy. Soc.* B, **106**, 229–50.

WIGGLESWORTH, V. B. (1938). The absorption of fluid from the tracheal system of mosquito larvae at hatching and moulting. *J. Exp. Biol.* **15**, 248–54.

WIGGLESWORTH, V. B. (1953). Surface forces in the tracheal system of insects. *Quart. J. Micr. Sci.* **94**, 507–22.

OXYGEN CONSUMPTION OF CASTRATED FEMALES OF THE BLOW-FLY, *CALLIPHORA ERYTHROCEPHALA* MEIG.

By ELLEN THOMSEN and KIRSTEN HAMBURGER

Zoological Laboratory of the Royal Veterinary and Agricultural College, Copenhagen, and the Laboratory of Zoophysiology, Copenhagen University

(*Received* 4 *April* 1955)

I. THE PROBLEM

In a previous investigation on the influence of the corpus allatum on the oxygen consumption of adult *Calliphora erythrocephala* (Thomsen, 1949), it was found that removal of the corpus allatum from the female fly had the effect that the oxygen uptake was lowered by 24%.

As allatectomy, when performed on a young *Calliphora*, also results in an inhibition of the growth of the ovaries (Thomsen, 1940, 1942, 1952), the author realized that the decrease of the oxygen consumption of allatectomized flies might be due to the inhibited growth of the ovaries. If this were the case the corpus allatum could not be said to have a direct effect on the oxygen uptake, but only an indirect one. This possibility might be tested by measuring the oxygen uptake of castrated females. The reason why such experiments were not undertaken at that time was that it was feared that the severe operation would interfere too much with the metabolism. Since then the operational technique has been improved so that by now it is only necessary to make a small incision in order to castrate the flies, and as will be seen in what follows, this incision does not influence the oxygen uptake.

II. TECHNIQUE

As in the former investigation, the oxygen consumption was measured by means of the Warburg apparatus, and the experiments were performed in the same way and at the same temperature, 25° C. (cf. Thomsen, 1949). It may be useful to stress the fact that, as in the previous experiments, each flask contained a single fly only. The flies used for the experiments were of the same age, i.e. 7 days (except in one case in which they were 8 days old), all the flies used in an experiment having emerged from the puparia within 2–3 hr. They were fed on meat, sugar and water and kept at 25° C.

The oxygen consumption of a total of 102 flies was measured. Of these thirty-seven flies were normal, unoperated females, twenty-nine were castrated females, and thirty-six were operated controls to the castrated females. Of the 102 experiments six were excluded because the flies were exceptionally active. One of these flies was a castrated one, two were operated controls, and three were normal, unoperated females.

In order to remove the ovaries two small incisions were made in the hind part of the third abdominal segment. The wound was sealed with paraffin wax, and care was taken that the neighbouring spiracle was not closed. Similar incisions were made in the operated controls, but the ovaries were not removed. In the case of the normal unoperated female a little paraffin wax was placed on the abdomen. (This was done because the oxygen consumption was expressed per unit of live weight.) The castration was undertaken when the flies were from 5 to 9 hr. old. In all, forty-one flies were castrated and twelve of these (29 %) died before the respiration experiment. Of the forty-seven operated controls, eleven (23 %) died. The mortality of the normal unoperated females was 33 %, but this was due to a very high mortality rate in one series only, caused by addition of too much water to the food. Since the flasks containing the flies were submerged in the water-bath, it was not possible to observe the flies continuously during the experiment. As a difference in the activity of the experimental animals and their controls might obscure the result, an attempt was made to measure the basal metabolism, as in the former investigation.

As it was most likely that the resting periods corresponded to the lowest readings, in each experiment the mean of the three lowest rates of oxygen consumption measured over 10 min. periods was accepted as a measure of the basal metabolism. In order to be reasonably sure of obtaining the lowest possible values, all experiments were excluded in which the difference between the three lowest readings of the manometer was greater than 2 mm. Thus since the readings of the manometers usually changed by about 10 mm. for every 10 min. period of 'basal metabolism', the three lowest readings were only accepted and averaged if they deviated by less than 20 %. In the previous paper 12 % of the experiments were discarded for this reason, ranging in a single experiment from 4 to 17 %. In the present work, the percentage of experiments which had to be excluded for this reason was 27 % for the operated controls, and 18 % for the normal, unoperated females; for the castrated flies the percentage was only 10. For the operated controls in particular the percentage excluded was very high, and on this account an additional set of calculations was made in which all the readings of each experiment were used. In these calculations the experiments were evaluated in the following way: the cumulative oxygen consumption measured over a 2 hr. period of time with readings every 10 min. was plotted against time. The average rate of oxygen consumption was calculated from the slope of the straight line which to the eye fitted the points best.

After the experiments the flies were dissected, and the developmental stage of the ovaries recorded. The outline of the corpora allata of the living flies was drawn, and the area calculated (cf. Thomsen, 1942, p. 334). Furthermore, the size of the fat-body was estimated.

III. EXPERIMENTS

(1) *Oxygen consumption of castrated females, operated controls and normal unoperated females*

The results of the experiments carried out under this heading are summarized in Table 1 and Figs. 1 and 2. It is quite obvious from the figures that there is no significant difference in oxygen consumption between the three categories. It is also to be noted that the results of the present experiments are in good agreement with the earlier work (Thomsen, 1949).

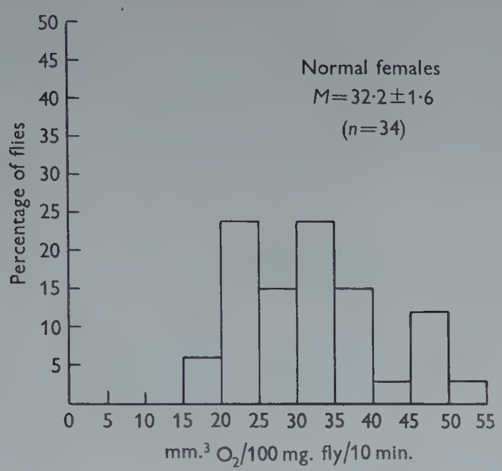
Table 1. *Oxygen consumption*

	mm.^{3} O_2/100 mg. fly/10 min. at 25° C., calculated from									
	A. All readings					B. Three lowest readings only				
	Max.	Min.	Mean	S.E.	No. of flies	Max.	Min.	Mean	S.E.	No. of flies
Normal females	50·5	17·8	32·2	±1·6	34	42·5	16·8	26·3	±1·4	28
Operated controls	47·6	18·4	33·5	±1·3	34	41·3	15·8	27·5	±1·4	25
Castrated females	47·6	18·4	32·9	±1·5	28	39·0	18·0	27·3	±1·2	26
Previously published data (Thomsen, 1949) for comparison										
Normal females	45·5	22·1	31·6	±1·2	26	38·6	21·2	28·3	±0·9	26

It may therefore be concluded that the oxygen consumption of female flies is not lowered by castration.

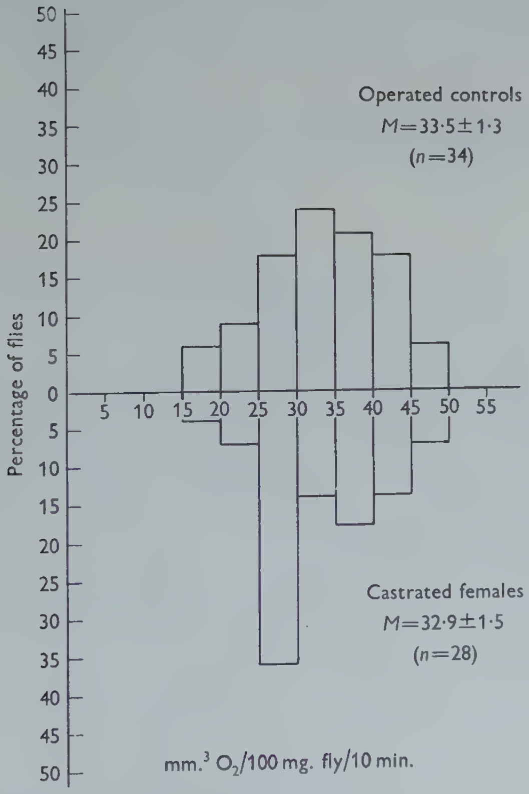
(2) *The size of the corpus allatum of the castrated females, the operated controls and the normal, unoperated females*

It was previously found that the corpus allatum of castrated females hypertrophies (Thomsen, 1940, 1942). This finding was corroborated in the present study. The figures for the castrated females and their operated controls are given in Fig. 3. The size of the corpora allata of the normal, unoperated females was of the same order as that of the operated controls. As removal of the corpus allatum resulted in a decrease in the oxygen uptake it might have been expected that females with enlarged corpora allata would have a high oxygen consumption; however, as the oxygen consumption of the castrated females—tending to have large corpora allata—and of the operated controls proved to be the same, it is not surprising that no correlation could be found when the size of the corpora allata was plotted against the oxygen uptake. This fact suggests that the hypertrophy of the corpus allatum might be due to a storage of the corpus allatum hormone rather than an indication of a higher activity of the organ.



Normal females
$M=32.2\pm1.6$
($n=34$)
Percentage of flies
50
45
40
35
30
25
20
15
10
5
0
5
10
15
20
25
30
35
40
45
50
55
mm.³ O₂/100 mg. fly/10 min.

Fig. 1.



Operated controls
$M=33.5\pm1.3$
($n=34$)
Castrated females
$M=32.9\pm1.5$
($n=28$)
Percentage of flies
50
45
40
35
30
25
20
15
10
5
0
5
10
15
20
25
30
35
40
45
50
55
mm.³ O₂/100 mg. fly/10 min.

Fig. 2.

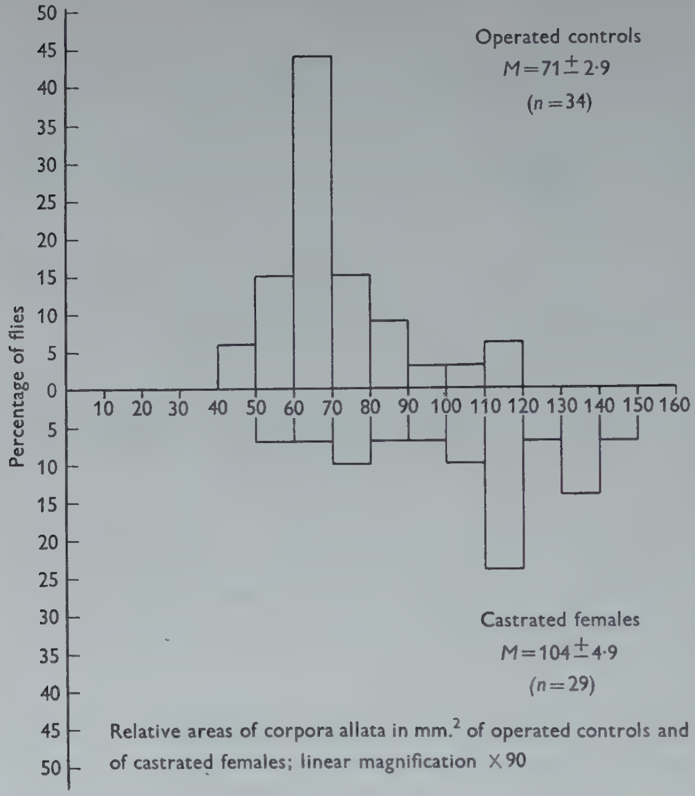


Fig. 3.

(3) *The fat-body of the castrated females, the operated controls and the normal unoperated females*

To our knowledge the only thorough investigation of the fat-body of a castrated insect has been made by Pfeiffer (1945), in her comprehensive paper on the effect of corpora allata on the metabolism of adult *Melanoplus differentialis*. Therefore, even if we have not as yet studied the effect of castration upon the fat-body of *Calliphora* females in detail, some rough estimates of the size of the fat-body of these females and their controls might be of some interest.

In Table 2 is given the estimated size and appearance of the fat-body of the castrated females and their controls. In this case the controls comprise both operated and unoperated controls. The group 'controls I' contains all the females with fully mature ovaries, whereas 'controls II' comprise the females in which for unknown reasons the development of the eggs was more or less delayed. From many dissections it has been found that even in females with mature ovaries the variation in the size of the fat-body is very great and this is also to be seen from the figures of Table 2 (controls I). The number of females with a hypertrophied fat-body (+ + +) is just as great as the number of females with a depleted fat-body (+), the majority being in between (+ +). In the castrated females, however, the number of flies with a hypertrophied fat-body (+ + +) is five times greater than the number of flies with a depleted fat-body (+). Furthermore, 21 % of the castrated females had a greatly hypertrophied fat-body (+ + + +), whereas none of the fat-bodies of the controls with mature ovaries belonged to this group. Admittedly the number of castrated females is rather small, but it looks as if they tend to accumulate more material in their fat-body than normal females.

Table 2. *Size of fat-body in castrated females and their controls*

	+ (%)	+ + (%)	+ + + (%)	+ + + + (%)	Total number of flies
Castrated	7	38	35	21	29
Controls I	28	44	28	0	53
Controls II	12	18	41	30	17

+ Fat-body depleted; + +, moderately enlarged; + + +, hypertrophied; + + + +, greatly hypertrophied. Controls I, females with fully mature eggs; controls II, females with eggs in different developmental stages, see text.

The group 'controls II' only comprises a small number of flies, and therefore one should be cautious in drawing any conclusions from the findings in this group. But it has been observed repeatedly that the fat-body very often hypertrophies when the development of the ovaries is delayed for unknown reasons. A similar tendency is seen in the flies of 'controls II'.

In all cases in which a hypertrophy of the fat-body was found, it was shown to be due to a great abundance of fat and in some cases of glycogen. A more thorough investigation of the fat-body is planned.

DISCUSSION

In the previous paper (Thomsen, 1949) there was some discussion as to whether the decrease in the oxygen uptake found in allatectomized females was due to the inhibited growth of the ovaries or whether it was due to the removal of a more general stimulating action of the corpus allatum on the tissues of the female fly.* However, normal unoperated females and males showed very nearly the same rate of oxygen consumption, and also allatectomy of the male resulted in a decrease in the oxygen consumption. It was therefore suggested that the influence of the corpus allatum on the metabolism of the female fly was most likely a general one.

This supposition has been corroborated in the present study, in which it was found that castration of females does not lower the oxygen uptake.

In accordance with this result no correlation was found between the magnitude of the oxygen consumption and the developmental stage of the ovaries.

How the corpus allatum exerts its effect on the respiratory metabolism cannot be explained for the time being, but must await further investigations. As to the influence of the corpus allatum on metabolism see Wigglesworth (1954, pp. 80–3).

---

* The decrease in the oxygen uptake of allatectomized females calculated on the three lowest values was found to be 24 %. If, however, the oxygen uptake was evaluated from the slope of the cumulative curves (see p. 693) the decrease only amounted to 16 %.

The tendency of the castrated females of *Calliphora* to contain rather a large amount of stored fat is in accordance with the finding of Pfeiffer (1945, p. 202) in *Melanoplus*. In *Calliphora* (Thomsen, 1942, 1952), as in some other insects, the fat-body of allatectomized females hypertrophies. Therefore the fact that castrated females may at the same time have an enlarged corpus allatum and a hypertrophied fat-body supports the view that the hypertrophy of the corpus allatum is due to a storage of hormone rather than to a higher activity (p. 694).

However, where the hypertrophy of the corpora allata is brought about by severing of the nervi corporis cardiaci, as in the experiments of Scharrer (1952) on *Leucophaea maderae*, the evidence is in favour of an increase in the activity of the corpora allata rather than of a decrease.

The experiments of Thomsen (1949) were discussed by Pflugfelder (1952, p. 248) who made the same comments as the author herself as to whether the effect of the corpus allatum on the oxygen consumption was most likely to be a direct or an indirect one. (Unfortunately Pflugfelder did not realize that the measurements of the oxygen uptake were made on single flies). Pflugfelder's data on *Dixippus*, mostly obtained on larvae, are somewhat difficult to compare with the data on adult *Calliphora*. The fact that in allatectomized *Dixippus* the oxygen uptake decreases just after the removal of the corpus allatum, but increases later on, is interesting because it indicates the possibility that some other organ might gradually take over the function of the corpus allatum. It is very natural to suspect that the pericardial glands or the ventral glands, or both, might be the responsible organs. In the adult *Calliphora* the side-lobes of the ring-gland (the peritracheal gland), homologous with the above-mentioned glands of *Dixippus*, degenerate during the first 3–4 days of the imaginal stage, so in the 7-day-old *Calliphora*, allatectomized when young (i.e. less than 8 hr. old), this organ cannot replace the function of the corpus allatum.

Quite recently L'Hélias (1954) has confirmed the results of Pflugfelder in so far that she found a decrease in the basal metabolism of the fifth stage of *Dixippus morosus*, allatectomized in the fourth stage, followed by an increase in the last stage.

The fact that the oxygen uptake of castrated females and of females with normally developed ovaries was found to be of the same order is in line with the view that the synthesis of the yolk material is performed somewhere outside the ovaries (presumably in the fat-body). This was originally suggested by Wigglesworth (1943), see also Pfeiffer (1945, p. 211). This idea is likewise favoured by Telfer & Williams (1952) and by Telfer (1954) who stated that a protein, antigen 7, of the ovaries of *Cecropia*, is probably synthesized by some tissue other than the ovaries.

In this connexion it is interesting that according to Clavert (1953) at least some of the yolk (protein and fat) of the bird's egg is synthesised in the liver and conveyed to the oocyte via the blood.

SUMMARY

1. The oxygen uptake of castrated females of *Calliphora* was measured and found to be of the same order as that of the 'operated controls', i.e. females operated upon in the same way except that their ovaries were not removed. This result confirms the conclusion drawn from previous experiments (Thomsen, 1949), viz. that the influence of the corpus allatum on the oxygen consumption works independently of the presence or absence of the ovaries.

2. However neither in castrated nor in normal females could any correlation be found between the size of the individual corpus allatum and the rate of oxygen consumption of the fly.

We want to thank Dr E. Zeuthen very much for many valuable discussions. It is a pleasure to thank the Carlsberg Foundation, which has supported this work with a grant.

REFERENCES

Clavert, J. (1953). La biochimie de l'ovogenese. *Arch. néerl. Zool.* **10**, 1 (Suppl.), 1–17.

L'Hélias, C. (1954). Étude du métabolisme basal chez le phasme *Dixippus morosus* après ablation des corpora allata. *C.R. Acad. Sci., Paris*, **239**, 778–80.

Pfeiffer, I. Weed (1945). Effect of the corpora allata on the metabolism of adult female grasshoppers. *J. Exp. Zool.* **99**, 183–233.

Pflugfelder, O. (1952). *Entwicklungsphysiologie der Insekten.* (*Probleme der Biologie* 5.) Leipzig.

Scharrer, B. (1952). Neurosecretion. XI. The effects of nerve section on the intercerebralis-cardiacum-allatum system of the insect *Leucophaea maderae*. *Biol. Bull., Woods Hole*, **102**, no. 3, 261–72.

Telfer, W. H. (1954). Immunological studies of insect metamorphosis. II. The role of a sex-limited blood protein in egg formation by the *Cecropia* silkworm. *J. Gen. Physiol.* **37**, no. 4, 539–58.

Telfer, W. H. & Williams, C. M. (1952). The relation of the blood proteins to egg formation in the *Cecropia* silkworm. *Anat. Rec.* **113**, no. 82.

Thomsen, E. (1940). Relation between corpus allatum and ovaries in adult flies (Muscidae). *Nature, Lond.*, **145**, 28.

Thomsen, E. (1942). An experimental and anatomical study of the corpus allatum in the blow-fly, *Calliphora erythrocephala* Meig. *Vidensk. Medd. dansk naturh. Foren. Kbh.* **106**, 320–405.

Thomsen, E. (1949). Influence of the corpus allatum on the oxygen consumption of adult *Calliphora erythrocephala* Meig. *J. Exp. Biol.* **26**, 137–49.

Thomsen, E. (1952). Functional significance of the neurosecretory brain cells and the corpus cardiacum in the female blow-fly. *Calliphora erythrocephala* Meig. *J. Exp. Biol.* **29**, 137–72.

Wigglesworth, V. B. (1943). The fate of haemoglobin in *Rhodnius prolixus* (Hemiptera) and other blood-sucking arthropods. *Proc. Roy. Soc.* B, **131**, 313–39.

Wigglesworth, V. B. (1954). *The Physiology of Insect Metamorphosis.* Cambridge Monographs in Experimental Biology, no. 1.

THE MECHANISM OF CELL DIVISION IN THE CLEAVAGE OF THE NEWT'S EGG

By G. G. SELMAN and C. H. WADDINGTON
Institute of Animal Genetics, Edinburgh

(*Received* 21 *March* 1955)

(With Plates 1 and 2)

CONTENTS

	PAGE
Introduction	000
Materials and methods	111
Changes in the shape of newt egg during cleavage	333
Movement of surface pigment granules during cleavage	444
Changes in elastic properties of the cortex during cleavage	555
Changes in subcortical morphology during cleavage	666
The interplay of the various factors	777
Discussion and comparison with cleavages in other forms	888
Summary	000

INTRODUCTION

Early work upon the nature and mechanism of the fundamentally important biological process of cell cleavage has been very adequately reviewed by Wilson (1928). In this book a broad distinction was made between 'cleavage by constriction', which is characteristic of cell division in higher animals, and 'cleavage by cell plate formation', characteristic of higher plants. In the former the furrow between the daughter cells may be seen to grow inwards from a peripheral zone, while in the latter the cell plate may be seen to form first inside the cell at a point intermediate between the daughter nuclei (e.g. Fujii & Yasui, 1954; Davis, Wilkins, Chayen & La Cour, 1954). In both cases the plane of cleavage is approximately at right angles to the spindle axis and across its largest cross-section. Indeed the idea that true cleavage is dependent, for its existence and location, upon a chromosome-bearing spindle (Boveri, see Wilson) is one of the very few early ideas which most subsequent work has tended to support rather than oppose. Boveri indeed showed (with invertebrate eggs or egg fragments) that centrioles with asters may multiply by periodic division in the entire absence of nuclei, chromosomes or spindles, and in these cases some irregular furrowing of the cell surface may occur. Such furrows usually do not penetrate far into the egg and fail to persist, so that Boveri did not regard them as valid examples of cleavage. On the other hand, cases may be quoted where the position of the cleavage furrow was modified by shifting the position of the anaphase spindle by mechanical deformation of the cell or by micromanipulation (Carlson, 1952; Waddington, 1952; Wilson, 1928, p. 159).

Instances of nuclear division occurring without cytoplasmic division are fairly common in nature, where they lead to multinucleate cells. For example, the first twelve nuclear divisions in the embryonic development of *Drosophila* are without cleavage (although centrioles, asters and spindles are present), after which the entire blastoderm cleaves synchronously, the cell walls growing inwards from the periphery. In the majority of cases, however, cleavage regularly follows nuclear division, as if a coupling mechanism exists between the two processes, which in certain circumstances does not operate.

Certain of the more recently advocated theories of cleavage still assign a dominant role to the asters. Gray (1924) considered that astral growth caused cleavage. Dan (1934) and Dan & Ono (1954), in order to explain their observations of surface movement in cleaving echinoderm eggs, consider that expansion of the spindle remnant pushes the asters against the polar surface to cause expansion there, while simultaneously pulling against the furrow surface to cause a local shrinkage. Yet cleavage has been shown to take place in nature in the absence of asters (cf. the study of pollen forming divisions in magnolia by Farr, 1918, and the cytology of coccids by Schrader, 1948), and may occur after the asters have been made ineffective by experimental procedures such as mechanical stirring (Chambers, 1938; Mitchison, 1953) insertion of a cellophane strip (Waddington, 1952) and the application of colchicine (Swann & Mitchison, 1953).

Very recent theories favour the view that cleavage is effected by expansions and contractions of the cell cortex. Dan (1954), while maintaining his original view of cortical contraction in the furrow region at the beginning of cleavage, asserts that the increase in cell surface, produced by cleavage, is finally accomplished by a local stretching or expansion of cortex in the furrow region. If the hyaline layer has previously been removed using calcium-free sea water this is a post-cleavage event. Swann & Mitchison (see Mitchison, 1952, 1953), again for the sea-urchin, consider that cleavage is initiated by a substance emanating from the daughter groups of anaphase chromosomes, which causes a wave of expansion in the cell cortex beginning near the spindle-poles and spreading to the equator where the furrow is formed by an active expansion of cortical material. The views of these authors are now slightly modified (see Mitchison & Swann, 1955).

Another explanation of cell cleavage is provided by Lewis (1939) and Marsland (1939, 1951), who maintain that cleavage is due to the contraction of a ring of gelated material in the furrow region.

In spite of the universal nature of cell cleavage, by far the majority of experimental studies have been made on early cleavage stages in echinoderms; and among these studies, work done after the hyaline layer has been removed by the use of calcium-free sea water has formed the larger proportion. Echinoderms undoubtedly offer a fine experimental material, but there would seem to be a need now for detailed studies to be made on different materials, so that a subsequent comparison of results may allow one to establish which are the fundamental characteristics of all cleavages, and which are peculiar to particular classes of cleavage. Thus cleavages in plants and animals might be compared with profit, as might holoblastic, extreme meroblastic cleavages, and intermediate types (see Nelsen, 1952). Cleavages in different types of animal tissues might also be compared with those of the egg.

MATERIALS AND METHODS

The early cleavage divisions of the newt egg offer several features of great value in any experimental study. The first is that of size. The *Triturus alpestris* egg is just over 2 mm. in diameter. This makes easier micromanipulation procedures (e.g. Waddington, 1952) and physical measurements. Secondly, the cleavages take place quite slowly (e.g. the first cleavage division in *T. alpestris* takes about 40 min. at 18° C.), which makes it easier to record the details of a particular cleavage on ciné film, and to perform measurements at intervals during its progress. Again, the newt egg possesses dark brown pigment granules embedded in its cortical layer by which surface movements may be followed. Lastly, the jelly capsule and vitelline membrane are easily removed by manipulation with fine forceps without any damage to the egg itself. It is always an advantage to be able to remove these membranes easily without having to resort to enzyme solutions, calcium-free media and the like, all of which may injure the egg surface; when observations are made with the aid of such media, it is always necessary to demonstrate that any conclusion drawn holds also for normal cleavage. The one-tenth strength buffered Holtfreter's medium is well established by many embryological studies as most suitable for the maintenance of natural development. The material has one major drawback. The large proportion of yolk in the cytoplasm renders it optically opaque and makes it impossible to parallel the many beautiful studies made on echinoderm eggs with polarized light (e.g. Swann, 1951*a*, *b*; Mitchison & Swann, 1952).

One material was used throughout the work described in this paper; that is the first few cleavages in the naturally fertilized egg of *T. alpestris*, studied in a medium of one-tenth strength Holtfreter's saline with a phosphate buffer at pH 7·0.

The more obvious features of cleavage in the newt are described in a large number of works on descriptive embryology. The cleavages are holoblastic. The first two are meridional and divide the egg into four equal blastomeres. They begin at the animal pole (where a slight puckering of the animal surface transverse to the forming furrow may usually be seen), and travel downwards from the pigmented animal surface through the yolky cytoplasm to the vegetative pole.

The analysis of cleavage, presented in this paper, was made possible by the use of three different kinds of experimental approach. In the first place measurements were made on ciné films taken by time-lapse microphotography by Mr Eric Lucey of this department. From these, the changes in shape of eggs during cleavage were followed, and the movement of the surface pigment granules was also recorded. Secondly, the changes in the elastic properties of the cortex were measured using an apparatus similar to that devised and used previously by Mitchison & Swann (1954*a*, *b*) for similar measurements with sea-urchin. Thirdly, permanent stained serial sections of eggs at particular stages of the cleavage process were examined for morphological changes in the interior of the egg. Finally, a theory is presented to provide an explanation for all the observations, and to show how cleavage takes place.

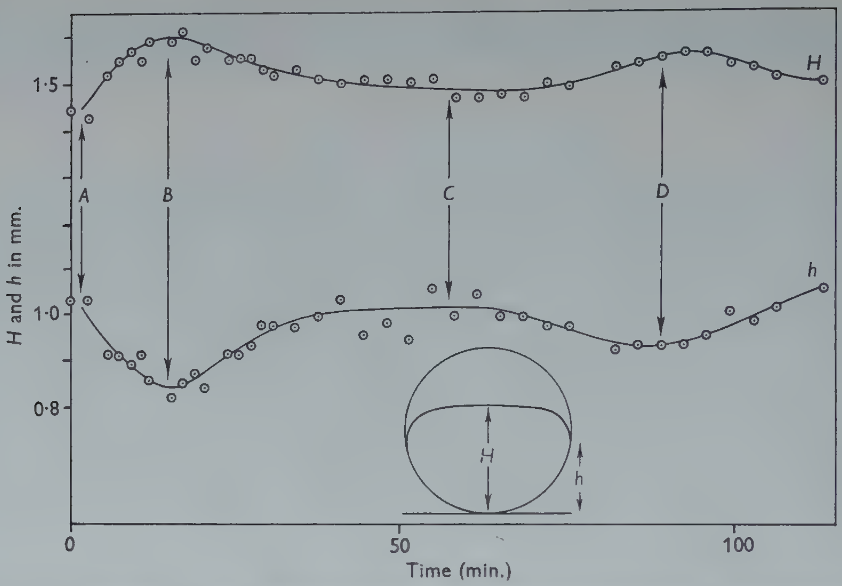
CHANGES IN SHAPE OF THE NEWT EGG DURING CLEAVAGE

When the jelly capsule is removed from a fertilized newt egg before cleavage, the egg may be clearly examined or photographed through the thin transparent vitelline membrane. The undamaged vitelline membrane is almost perfectly spherical. (In fact it is an ellipsoid whose vertical axis of revolution is about 92% of any horizontal diameter.) The egg itself occupies only the lower two thirds of the intra-vitelline space (Text-fig. 1), so that whereas the vegetative surface is constrained to follow the almost spherical shape of the vitelline membrane, the animal surface, which is always uppermost, shows a pronounced flattening due to gravity. The surfaces of both the egg and the vitelline membrane are smooth. Free movement of the egg surface is not apparently restrained by its contact with the inner surface of the vitelline membrane, as may be demonstrated by inverting the egg within the vitelline membrane, after which it rights itself in a few seconds. After complete removal of the vitelline membrane the egg slowly settles down on a flat surface to a rather flat bun-shape, the equilibrium position being reached in about a minute or two (Text-fig. 2). In this the egg behaves like a rather viscous liquid drop bounded by an elastic cortical layer. When the egg has fully settled down, its shape is governed by an equilibrium between gravitational forces acting on the fluid bulk of the egg (which has a specific gravity of about 1·08) tending to flatten it into a disk, and the elastic forces in the surface layers which tend to return the egg to a more spherical shape. Cleavage proceeds normally, within or without the vitelline membrane, the only real point of difference being that a rather greater area of new cortical material is exposed on the surface in the furrow region in the latter case.

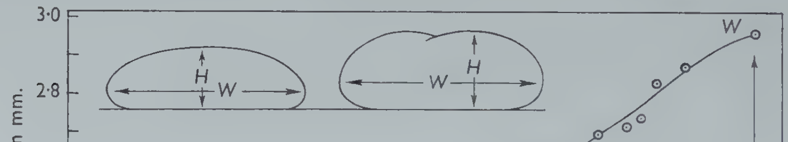
The thickness of the elastic cortical layer is roughly estimated from fixed and sectioned eggs to be 2μ. This estimate is facilitated by examining eggs which have been *lightly* centrifuged before fixation (see later section). Those pigment granules in the more sol-like interior of the egg are then shifted from sites near the animal surface towards the centrifugal pole, whereas those granules embedded in the more gel-like cortical layer remain.

When the surface of an egg is punctured with a needle, it does not crumple like a punctured football bladder (as the internal turgor if any is released). From a small puncture only a slight amount of yolk flows out slowly, and the wound is later closed by a characteristic healing process described by Holtfreter (1943), in which the coat which is the surface layer of the cortex, contracts round the damaged region to seal it off.

Miss H. Yates, of this department, measured the rate of movement of the externally visible furrow across the surface of eggs, using a microscope with micrometer eyepiece and prisms for side viewing. For eggs cleaving within the vitelline membrane, the furrow was found to move from the animal to the vegetative pole at a



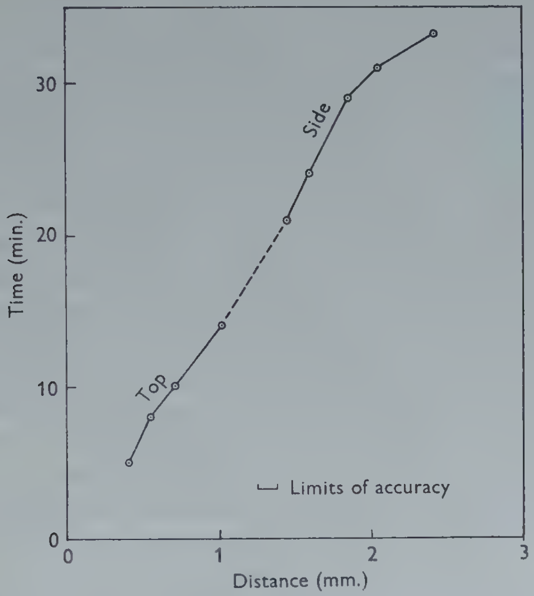
Text-fig. 1. The graphs indicate the changing shape of an egg cleaving within the vitelline membrane. Measurements *H* and *h* (see diagram of egg) were made from ciné film showing the egg in elevation. Times *B* and *D* correspond to the beginning of first and second cleavage respectively. *A* was before first cleavage and *C* midway between first and second cleavage.



Text-fig. 2. The graphs indicate the changing shape of an egg cleaving on a flat agar surface after the vitelline membrane had been removed. Measurements *H* and *W* (see diagram of the egg) were made from ciné film showing the egg in elevation. Times *A* to *B* to *C* were before first cleavage when the egg was 'rounding-up'. At *C* the first sign of the 'dipping-in' at the animal pole was seen. At *D* the furrow was across the animal surface. At *E*, cleavage was complete.

fairly constant rate: the mean value for fourteen measurements was 0·061 ± 0·016 mm./min. during first cleavage at 17·2 ± 0·6° C. The progress of the furrow in a typical case is shown in Text-fig. 3.

Successive frames of ciné films taken to show newt eggs, in elevation, cleaving within the vitelline membrane, demonstrate that the animal surface of the egg reaches a more spherical shape at the moment when the cleavage furrow first appears at the animal pole. The egg subsequently 'relaxes' its shape and is at its flattest roughly midway between one cleavage and the next. This alternate 'rounding-up' and relaxing movement of the egg during cleavage is repeated with each cleavage and has been followed on a projected ciné film from first cleavage to the morula stage. Measurements were made of the height (H) of the animal pole of the egg above the vegetative pole, and of the height (h) from the level of the vegetative pole to the level of the contact-point between the side of the egg and the vitelline membrane, using enlarged prints from frames spaced at regular intervals along a film showing a cleavage sequence. The results, plotted against time (Text-fig. 1), illustrate the changing shape of the egg during first and second cleavage.



Text-fig. 3. The graph shows the progress of a first cleavage furrow across the animal surface and down the side of an egg cleaving within the vitelline membrane at 17° C.

This behaviour can be observed even more readily in cleaving eggs which are observed in elevation after they have been freed from the vitelline membrane and placed on a flat agar surface. A series of outline diagrams was traced from a film to show the 'rounding-up' process during first cleavage. Again the maximum height of the egg was found to be at the moment when cleavage began, and the movement is shown by a plot of the height (H) and the maximum diameter (W) of the egg, against time (Text-fig. 2), from measurements made on a film sequence taken during first cleavage.

Harvey & Fankhauser (1933) have interpreted similar observations of changes in shape during cleavage of eggs of the salamander *Triturus viridescens* in terms of 'apparent surface tension'. Using similar methods to theirs our measurements give similar values. However, the egg surface is not like a liquid/liquid interface. The surfaces of eggs contain complicated structural elements (e.g. see Mitchison, 1952), and the behaviour of these when stretched is unlikely to follow the formulations applicable to the surfaces of liquid drops. Although the shape of an egg does superficially resemble a liquid drop, it will be shown in a later section to behave differently under deformation.

The changes in shape of the egg during cleavage are those to be expected from a consideration of the mechanical model of the egg to which we have already referred, If towards the beginning of cleavage there were an increase in the value for the Young's modulus of the elastic cortical layer, or alternatively an increase in its thickness, then the egg would be flattened less easily under gravity and would 'round-up' to assume an equilibrium position more closely approximating to a spherical form.

Using this same model, and assuming the elastic cortical layer to be isotropic, it is theoretically a matter of calculation to derive a value for the Young's modulus of the cortex from measurements on the shape of the egg and using estimates for the thickness and Poisson's ratio of the cortical layer. However, apart from the fact that the cortical layer is almost certainly not isotropic (it would be expected to have different elastic moduli for stresses within the plane of the surface than for directions normal to it), the calculation itself is not straightforward and we have not succeeded in doing it. Fortunately the experimental conditions of the measurements on the elastic behaviour of the cortical layer, described in a later section of this paper, lend themselves more easily to calculations of values for Young's modulus.

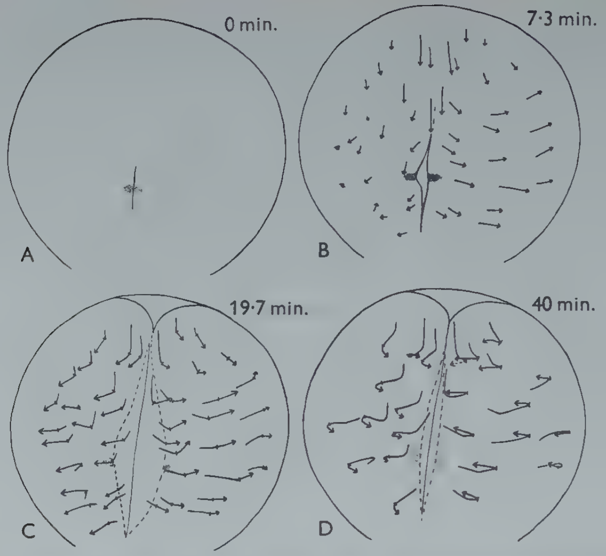
One particular film sequence, from which the illustrations in Pl. 1, figs. 1–4, were taken, clearly showed 'rounded-up' positions during cleavage in which the height of the individual blastomeres was greater than their horizontal diameters. Such behaviour cannot be accounted for merely by postulating changes in thickness or elastic properties of the cortical layers; some additional factor is involved. The behaviour is explained by the formation of new cortical material growing out of the cleavage furrow region (see later).

MOVEMENT OF SURFACE PIGMENT GRANULES DURING CLEAVAGE

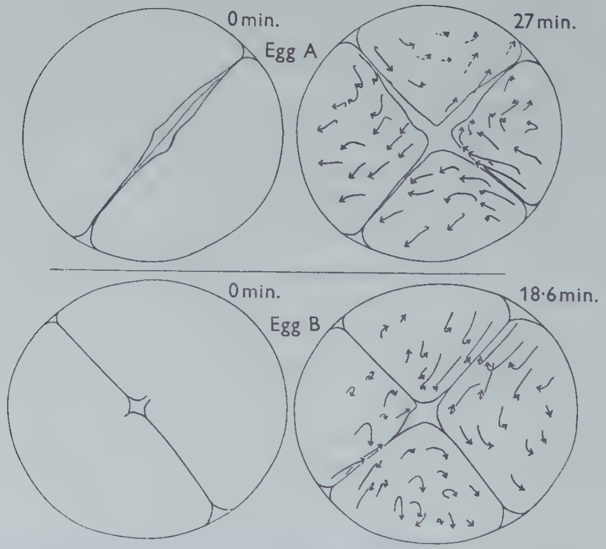
When a number of film sequences of eggs cleaving within the vitelline membrane were projected, it was found that the clusters of pigment granules on the animal surface could easily be resolved and their movement followed on the screen. By tracing the movement of individual clusters with a pencil during cleavage, using markers fixed at intervals along the film to indicate passage of time, a diagram showing the movement of the entire animal surface of the egg was built up. The pigment granules, whose movements were thus observed, are embedded in the surface layers of the cortex (i.e. the coat), in gel of considerable rigidity; this can easily be demonstrated by micromanipulation. It is justified therefore to regard the movement of the granules as indicating movement of the surface cortical layer in which they are embedded. During the cleavage sequence the egg rests upon its vegetative surface within the vitelline membrane. Sometimes during the filming, the egg may rotate or rock slightly about its vegetative pole. Such a movement is easily detected and measured by observing its effect upon the position of the outline of the egg or a cleavage furrow. The effect of such a movement of the whole egg upon the observation of relative movements of the pigment granules with respect to each other and the forming furrow, is to complicate the diagram unnecessarily. In such cases, a correction was applied, using a vector method to subtract the movement which each pigment granule possessed as a result of the movement of the whole egg from the total observed movement. It is important to note that this correction does not alter in any way the relative motion of the pigment granules with respect to each other; nor does it alter their movement with respect to the position of the forming furrow. All the illustrations of pigment movement in this paper (Text-figs. 6–8) were taken from film sequences of cleavage in which the correction for the movement of the whole egg was a small or negligible fraction of the total movement observed on the screen. All the movement in the diagrams took place during cleavage; between cleavages little or no movement of pigment granules could be seen.

Detailed diagrams showing the paths of pigment granules during seven cleavages were observed in this way. All showed a similar pattern of behaviour. Cortical movements are seen to be greater near the forming furrow and least on that part of the egg surface remote from the furrow and opposite the poles of the spindle.

When viewing the egg from directly above the animal pole, the beginning of cleavage is seen to be accompanied by a movement of cortex along the line of the future furrow, from the sides of the egg toward the animal pole at which the furrow first appears. This takes place in the first 5–7 min. of cleavage, and it is obvious in all films of first or second cleavage showing the animal surface. It accompanies the 'dipping in' of the new furrow at the animal pole, and the temporary appearance of the tiny wrinkles in the coat transverse to the forming furrow. At the same time, the pigmented cortex on either side of the forming furrow begins a slower movement in a direction away from and at right angles to the furrow. The furrow thus opens out in its widest extent, which is reached about 20 min. after the beginning of first cleavage, when white unpigmented cortex is revealed within the new furrow and the furrow has reached rather more than half way round the outside of the entire egg. The line of demarkation, between the pigmented animal surface on either side of the furrow and the unpigmented cortex within it, is clearly defined (see Pl. 1, fig. 7, and the position of dotted lines in Text-fig. 4*c*). There is no sign of a local cortical movement into the furrow such as Schechtman (1937) has considered to be the immediate cause of cell division in newt; in fact the movement is in the opposite direction. During the final 20 min. in which cleavage is completed, the pigmented cortex on either side of the furrow moves slowly back towards it again, and the white unpigmented cortex within the furrow retreats beneath the surface to form



Text-fig. 4. Four diagrams traced from projected ciné film to show movement of pigment on the animal surface of an egg during first cleavage within the vitelline membrane. In each diagram are shown the complete courses traced out by pigment granules since the beginning of the cleavage. In C and D the distance between the arrow-tip and the mark across the length of the arrow indicates the path traced out since the time of the previous diagram. In B the arrow length indicates the entire path of the pigment since A.

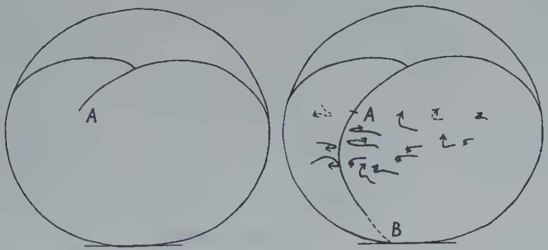


Text-fig. 5. Diagrams of the courses traced out by pigment on the animal surface during the entire second cleavage period for two different eggs cleaving within the vitelline membrane.

part of the cortex by which the daughter blastomeres remain in contact after cleavage.

When an egg is viewed by ciné technique to give a side view of the egg as the furrow moves down to the vegetative pole (see Text-fig. 6), the pigment is seen to move towards the forming furrow, and then away from it. The movement is clearly larger near the furrow and very small on the surface opposite the spindle poles.

The path which any individual pigment granule traces out on the surface of an egg during cleavage is thus largely cyclic, and the distance between the initial and final positions is usually much smaller than the total length of the path traced. Such net translational movement as does take place is in a direction toward the animal pole for regions toward the sides of the egg and in line with the forming furrow; it is away from the furrow for pigmented regions on top of the egg on either side of the furrow, and it is toward the furrow region, for cortex at the sides of the egg nearer the vegetative pole. The translational movements themselves thus make up a cyclic pattern on the surface of the egg, which means that during a cleavage a large change in net surface area of pigmented cortex or coat is not to be expected.



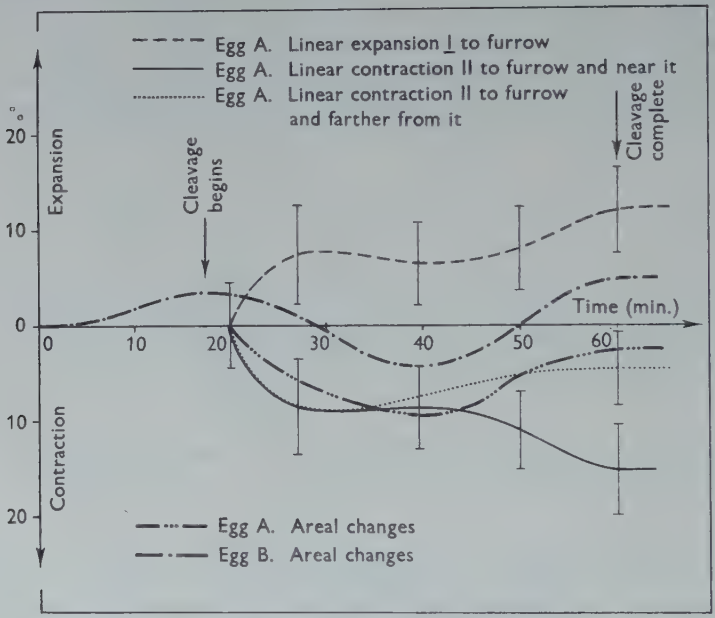
Text-fig. 6. Diagram of surface pigment movement on the side of an egg during first cleavage, while the furrow progressed from the point *A* to the vegetative pole *B*.

However, it is only fair to test hypotheses of cleavage which depend upon expansions of the cortex which exists before cleavage by measuring linear and areal expansions. This was done for the pigmented cortex on either side of the forming cleavage furrow, where the animal surface of the egg was fairly flat.

The mean percentage changes in distance between pairs of pigment granules were calculated for specific regions on the egg surface, and for measurements made in particular directions across the egg. All measurements were made using the film sequences showing the pigment movement in the greatest detail.

The linear expansions and contractions plotted against time (Text-fig. 7) show the behaviour of the animal surface of a particular egg; these results are means of 144 individual measurements made between pairs of pigment granules. The results for other eggs were similar, in both first and second cleavages. During the entire cleavage process, there is approximately a 10% linear increase of the animal pigmented surface on either side of the furrow for measurements made in directions perpendicular to the furrow. At the same time and place, a 10% linear decrease is observed, when measurements are made in directions parallel to the furrow. The total linear decrease which takes place is slightly greater than 10% when measured for the pigmented surface adjacent to the furrow, and rather less than 10% when measured along lines further away from the furrow. Areas of the cortex thus change their shape during cleavage, and this has also been shown by staining areas of the cortex with vital dyes.

Areal measurements were made using a planimeter on the prepared diagrams of pigment movement. The areas were outlined by as many pigment granules as possible, and were roughly of equal breadth and width. The areas did not include the unpigmented regions of the furrow, and were chosen to be either on those parts of the animal surface which were fairly flat, or on the sides of the egg where the curvature did not alter during cleavage because the egg surface was in contact with the vitelline membrane.



Text-fig. 7. Graphs of linear and areal changes measured for the animal surface of the egg for which the pigment movements in Text-fig. 4 were recorded.

The percentage changes in surface area, for pigmented regions on the tops of the two eggs during first cleavage, are plotted against time in Text-fig. 7. It will be seen that the final area does not differ from the initial area by more than 5% in either case.

In Table 1 are shown the percentage areal changes of the pigmented cortex in regions where pigment granules could be used to define areas with sufficient accuracy. Where the areas to be measured are changing shape, it is essential to outline the areas by as many points as possible round the perimeter. If the areas are poorly defined the measurements become very unreliable, and the areal results of Dan & Ono (1954) for sea-urchin would appear to be open to criticism in this respect. It will be seen from the table, that where measurements show that a significant expansion has taken place on one side of the furrow (e.g. an expansion of the order 10 %), then in every case a corresponding contraction will be found to have taken place on the other side of the egg. This behaviour could have resulted from a slightly unequal division of the original pigmented cortex between the two blastomeres. Where measurements were made for areas on each side of the cleavage furrow, an average of the percentage changes was taken. For second cleavages, the average was taken of the measurements made on the animal surfaces of each of the four blastomeres. In no case did this average deviate significantly from zero. There is thus no evidence for any net change in surface area of the pigmented surface during cleavage.

Table 1. *Changes in area of pigmented cortex during cleavage*

Cleavage	Egg	View	Side of furrow	No. of granules round area	% areal changes	Average areal change (%)
1st	A	Top	Left	15	− 7·3	− 2·95
		Top	Right	20	+ 1·4	
1st	B	Top	Left	7	+22·8	+ 4·9
		Top	Right	6	−13	
1st	C	Side	Left	10	+13	+13
2nd	B	Top	Left 1	9	−15·2	− 1·75
		Top	Right 1	11	−13·7	
		Top	Right 2	9	+21·9	
		Top	Left 2	5	0	
2nd	D	Top	Left 1	14	+ 5·9	+ 1·9
		Top	Right 1	8	− 9·3	
		Top	Right 2	11	−21	
		Top	Left 2	10	+32	
2nd	C	Side	Left	5	− 6	− 6
3rd	C	Side	Left	6	+13	+ 1
		Side	Right	5	−11	
					Mean of all eggs	+1·4±5·7 %

Now it is known from geometrical considerations that if an egg cleaves to give two equal daughter blastomeres, each of similar geometrical form to the original egg, then a 26 % increase in surface must occur. Where the daughter blastomeres do not separate completely but remain with plane surfaces in contact, as in the newt, the increase in surface resulting from cleavage is slightly greater than this. For the first cleavage in the newt within the vitelline membrane, the increase required would be at least 30 %, of which all but about 2 % would be required to form the wall between the cells beneath the surface of the egg.

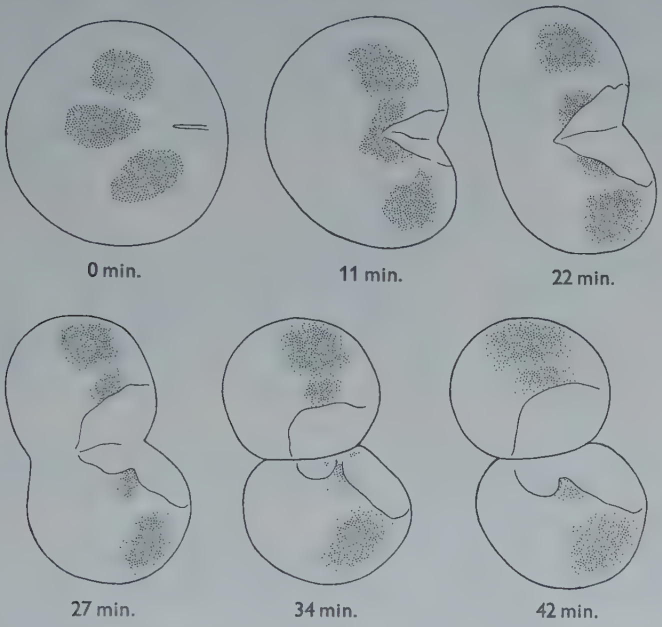
This large increase in area of cortex, to be provided during the first cleavage, is only to be reconciled with our conclusion that the total area of the original pigmented cortex does not expand at all during cleavage, by assuming that an additional area of new cortex is synthesized during cleavage in a region where we could not observe it; that is in the furrow region beneath the surface of the egg. The only possible basis upon which we could retain Schechtman's hypothesis that localized growth of the original cortex on either side of the furrow produces cleavage, would be to assume that the growing region was a band round the egg which extended less than 50μ on either side of the furrow, and which increased in area during cleavage at least ten times. It would not be sufficient merely to assume an expansion of this area, because the expanded surface would be then so thin that it could not possibly possess the mechanical rigidity demonstrated in the next section. Presumably this enormous increase in area would so dilute the pigment that it would appear white when it subsequently appeared in the furrow region 20 min. after cleavage had begun. Our interpretation will be seen later to be rather different from this.

When newt cleavage takes place on a flat surface with the vitelline membrane removed, the increase in surface area is about 27 %, but since an 11 % increase is sufficient to form the plane surfaces by which the blastomeres remain together, another 16 % increase must be provided for the outer surface. The new unpigmented cortex produced in the furrow region is in this case required to cover part of the outside of the egg, and for this reason the unpigmented cortex in the furrow region is considerably more conspicuous. Seen from above, the plan outline of the egg changes from a circle before cleavage to a figure of eight when it is complete. There is thus considerable translational movement of the daughter blastomeres, away from the furrow, as the blastomeres fall apart under gravity with the completion of cleavage. Otherwise the behaviour and motions of pigmented cortex are similar to cleavage within the vitelline membrane. The translational movement considerably reduces the accuracy of any areal measurements which may be made on eggs after the vitelline membrane has been removed.

It will already have been noted that the magnitude of the linear changes between pigment granules in the cortex of newt is incomparably smaller, relative to the dimensions of the egg, than has been observed for cleavage in echinoderms. Dan, Dan & Yanagita (1938) and Dan & Ono (1954) report linear expansions of 50 and 100 % between kaolin particles embedded in the cortex on the same side of a forming cleavage furrow, and although the changes are greatest near the furrow, even in the regions opposite the spindle poles linear expansions of 20 and 30 % are recorded. The corresponding areal estimates are also very large.

If, using our data for a newt egg cleaving within the vitelline membrane, measurements between pigment granules located on opposite sides of the cleavage furrow are made during the first 20 min. of first cleavage, then indeed linear and areal increases of several hundred per cent may be recorded. Such results would not of course indicate a corresponding expansion or growth of the cortex, since in that period of time the new unpigmented cortex (which as we show later has been formed beneath the surface of the egg) is being progressively revealed in the furrow region as the daughter blastomeres fall apart on either side.

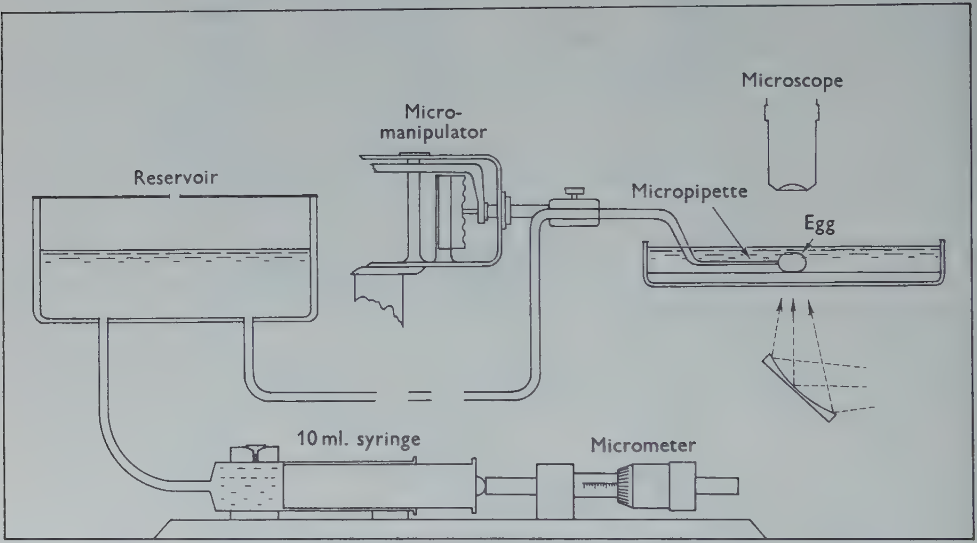
A number of experiments were performed by Miss H. Yates, using a local vital staining method. The vitelline membrane was torn above the animal surface of the egg before cleavage, and strips of agar impregnated with Nile Blue were inserted and left for about 30 min. so that patches of the surface cortex were vitally stained. During cleavage, the positions of the stained patches were followed by making drawings using a camera lucida. The surface movements, which were recorded by this method, were always similar to those observed from the behaviour of pigment granules, but measurements were not made because the dye showed a tendency to spread through the cortex so that the outline of the stained areas became more diffuse with time. The series of drawings shown in Text-fig. 8 support the conclusion that the white surfaces exposed in the furrow region during mid-cleavage has been newly synthesized in a subcortical region. The drawings relate to an egg whose vitelline membrane has been removed, so that rather more white surface is exposed in the furrow region on the animal surface than would be the case with the membrane intact. When cleavage is almost complete, a local tendency for a small part of the pigmented coat to spread into the furrow region is shown; this movement is subsequently reversed.



Text-fig. 8. Camera lucida drawings to show the movements during first cleavage of three areas of cortex which had been vitally stained after removal of the vitelline membrane.

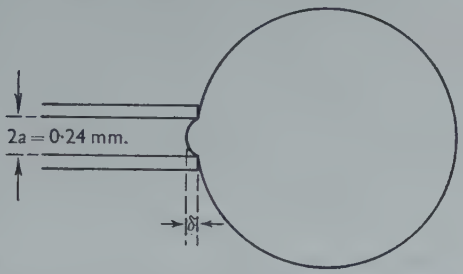
CHANGES IN ELASTIC PROPERTIES OF THE CORTEX DURING CLEAVAGE

These measurements were made using an apparatus and method similar to that previously devised and used by Mitchison & Swann (1954*a*) for measurements on sea-urchin eggs. These authors call their apparatus a cell elastimeter, for it is used to record changes in the elastic properties of the egg cortex. Our form of the apparatus is shown diagrammatically in Text-fig. 9. After the jelly capsule and vitelline membrane had been removed from the egg, it was allowed to rest on an agar surface in 0·1 strength Holtfreter's saline in a Petri dish upon a microscope stage. The Pyrex glass micropipette was drawn out to give an internal diameter at the tip of 0·24 mm. The wall thickness of the glass at the tip was approximately equal to the internal radius, and the tip was fine ground to give a flat surface perpendicular to the horizontal length of the pipette. Such a surface was found to give an excellent hydrostatic seal when apposed to the side of the egg. The micropipette was mounted on a Fonbrune pattern micromanipulator, and connected by a flexible tube to a cylindrical reservoir of 7·5 cm. diameter in which the saline level was approximately equal to that in the Petri dish. Fine adjustment of the hydrostatic level in the reservoir was made using a 10 ml. syringe fitted with a micrometer adjustment.



Text-fig. 9. Diagram of the apparatus used to measure the changes in elastic properties of the egg cortex during cleavage.

In making a measurement, the top of the pipette was first placed alongside the egg, opposite but not touching the particular point of the egg surface where the measurement was to be made. Using the micrometer adjustment, the level of the saline in the reservoir was then adjusted until an equilibrium position was reached when water neither flowed in nor out from the tip of the micropipette. The flow of water inside the micropipette was observed by focusing the microscope on minute fat droplets included in the saline. When no flow was observed, the tip of the micropipette was gently pressed against the side of the egg, using the fine horizontal movement on the micromanipulator. Since the diameter of the micropipette was small compared with the diameter of the egg, the surface of the egg then made an almost flat seal at the end of the tube. The hydrostatic pressure within the micropipette was then reduced a small known amount (p) by withdrawing a small known volume of saline from the reservoir of known surface area. It was then necessary to wait at least 5 min. until the egg surface attained an equilibrium shape within the the pipette. During this time the fat droplets were observed and checked for zero movement. Any movement would indicate a poor seal and the measurement would have to be discarded. At equilibrium the deformation (δ) of the egg surface down the middle of the pipette was recorded using a scale in the ocular of the microscope (Text-fig. 10). Finally the pressure within the micropipette was increased to its former value, the pipette moved horizontally away from the egg, and the zero equilibrium position checked. Any appreciable lack of agreement with the initial equilibrium value, due to evaporation from the Petri dish for instance, would lead to the result having to be discarded.



Text-fig. 10. Diagram of a deformation on the egg surface, produced by a reduction in hydrostatic pressure p within the micropipette, as viewed by the observer down the microscope.

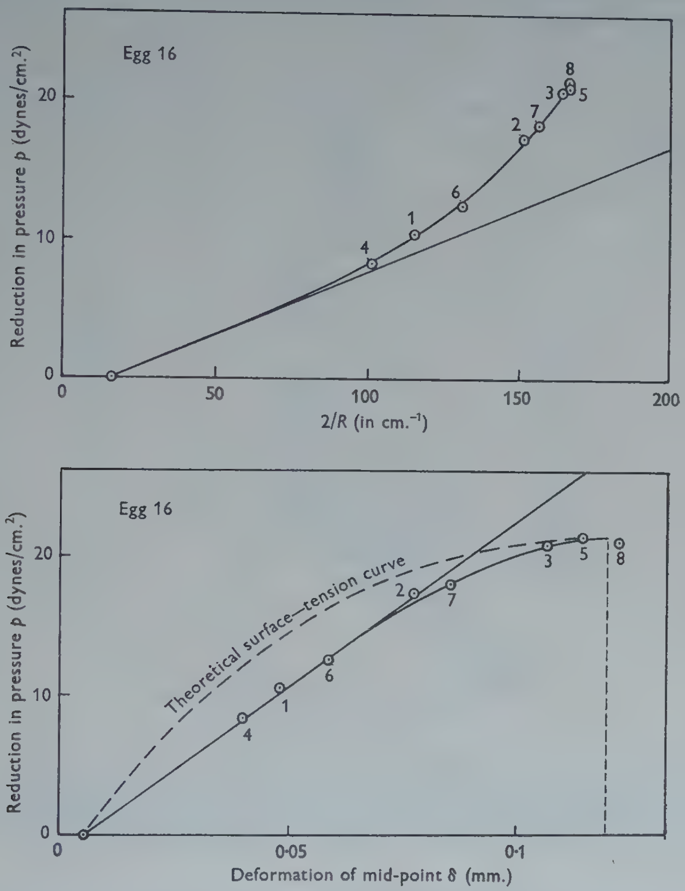
In practice it was found that the flat fine-ground finish of the rather thick-walled micropipette enabled perfectly reliable seals against the surface of the egg to be made without difficulty. In this way, provided the elastic properties of the cortex did not vary during the time the measurements were being made, a series of corresponding values of negative hydrostatic pressure (p) and mid-point equilibrium deformation (δ) could be recorded. The measurements were limited to those values of p for which δ was less than the internal radius of the micropipette. If greater reductions in pressure (p) were applied, the egg was sucked steadily up the tube and was usually destroyed in the process.

When a negative pressure (p) had been applied, during the course of a measurement, the equilibrium value for deformation (δ) was approached exponentially. If, after an appreciable deformation had been produced, the pressure was rapidly increased again to the equilibrium value and the pipette withdrawn, then the shape of the deformed surface was left impressed like a pimple on the egg surface; and thereafter its height also diminished exponentially. The relaxation time, associated with either the decrease or increase of δ, was of the order 3 min. for most eggs before cleavage or after cleavage, provided that the measurement was made on the animal surface or at the side of the egg. At the vegetative pole the relaxation time was rather more than twice this value. Eggs which had been stored in the refrigerator also showed large relaxation times. That equilibrium was not attained more quickly, was due therefore to the 'viscosity' of the cytoplasm, which would be greater at the vegetative pole, and would increase on refrigeration. If the cortex possessed plastic in addition to elastic properties, then relaxation effects would again be introduced (Tyler, 1942), but these would not be expected to change towards the vegetative pole. Whatever the contributory causes of the relaxation effect, if the corresponding values of p and δ are to be used to record changes in the elastic properties of the cortex then it would seem essential to use equilibrium values only, even although the rate at which the measurements can be recorded is thereby diminished. Only measurements at equilibrium were used in this study.

Measurements made on the same fertilized egg, between 2 and 3·5 hr. before the first cleavage, are shown in Text-fig. 11. The order in which the equilibrium measurements were made is indicated by numbers. All the measurements lie on a single smooth curve, because during this stage the elastic properties of the cortex do not vary with time. The line is straight for small deformations, but departs from linearity for larger values. This departure from linearity is very probably due to the egg surface slipping round the edge of the micropipette, and this probably does not occur for smaller deformations where the curve is linear. This conclusion may be justified by an experiment in which a large equilibrium deformation is first made (e.g. a value similar to point 4 in Text-fig. 11 is obtained) and the corresponding negative pressure is then halved, without first returning the pressure to zero. When equilibrium has again been attained, this reading will be found to lie below the normal curve (i.e. we have been tracing out part of a hysteresis loop). If the experiment is afterwards repeated, but with the initial deformation on the linear part of the graph (e.g. like point 6 in Text-fig. 11), then the subsequent half value will be found to lie on the straight line and not below it. This behaviour is consistent with a state of affairs in which slipping only occurs over the edge of the micropipette for the larger values of reduced pressure which are sufficient to overcome the frictional forces associated with the coat slipping over the ground glass surface. If these views are correct, then the slope of the linear part of the curve will be proportional to the Young's modulus of the cortical layer of the egg, and also proportional to the third power of its thickness. Measurement of the slope of the linear part of the curve will therefore give a measure of the resistance to flexure on the part of the surface cortex.

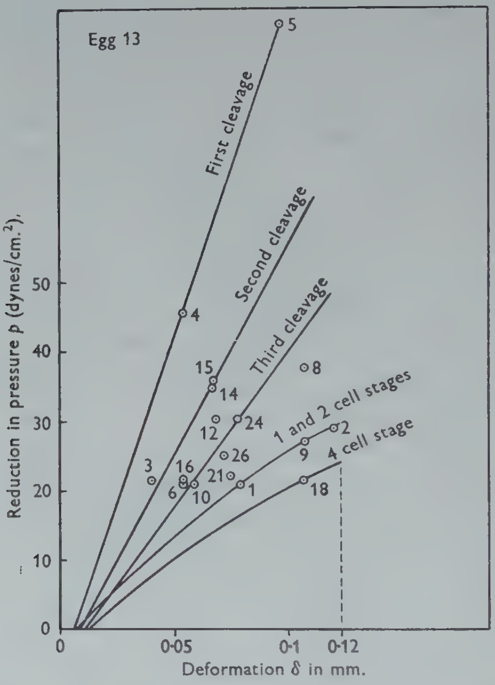
If we assume the deformations to be spherical in shape we may replot the observations in terms of the curvature ($2/R$) produced by the applied negative pressure (p) where $2/R = 4\delta/(\delta^2 + a^2)$, and where R is the radius of curvature produced within the pipette of radius a. The resulting curve is shown in Text-fig. 11, and the fact that it is not linear implies that the surface layers of the egg do not behave under deformation like the surfaces of liquid drops. The 'apparent surface tension' concept is therefore inapplicable here. On the contrary, the egg surface behaves like a solid layer possessing elasticity.

It was found that precisely similar values were recorded for measurements made on any part of the egg surface during similar periods with respect to the cleavage cycle. This result was true for eggs before cleavage, during cleavage and after cleavage. The results did, however, vary with time during cleavage. Measurements were made on fifty eggs during cleavage stages. When the results were plotted as equilibrium deformation (δ) against the negative hydrostatic pressure (p) required to produce it, the results always lay on curves which were similar in form to that shown in Text-fig. 11, but whose slopes varied with time in a regular manner during the cleavage cycle. The curves were however always linear for the smaller values, and the variation of their slopes with time during cleavage was measured.



Text-fig. 11. Graphs of mid-point deformation δ and curvature $2/R$, plotted against the reduction in hydrostatic pressure needed to produce the deformation, for a fertilized egg at 18° C. between 2 and 3½ hr. before first cleavage. The numbers on the points indicate the order in which the measurements were made.

A series of measurements made upon a single undamaged egg, while it developed at 18° C. from an uncleaved egg until third cleavage, is shown in Text-fig. 12; the order in which the measurements were made is indicated by numbers. The curves which have been constructed are those with maximum and minimum slopes during the period. Measurements lie on a line of minimum slope for eggs more than 40 min. before the first cleavage and when a cleavage has just been completed. As cleavage approaches, the individual measurements lie on curves of gradually increasing slope, until the moment when cleavage may just be seen to have begun, when they fall on the line of maximum slope. Thereafter until cleavage is finally complete, the measurements lie on lines of decreasing slope. This cycle of events is repeated for each successive cleavage.

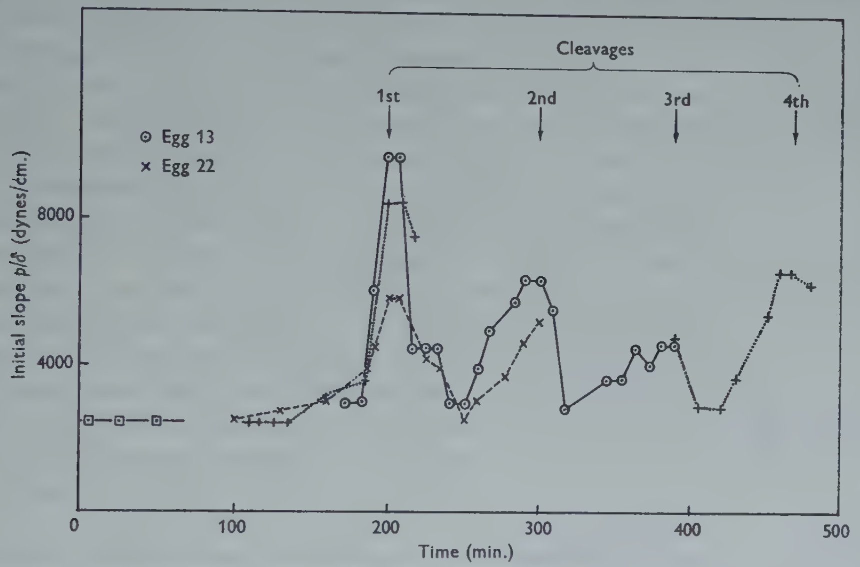


Text-fig. 12. Measurements of mid-point deformation δ and corresponding values of pressure reduction p, for a single egg during its first three cleavage divisions. The numbers indicate the order in which the results were obtained. The drawn curves are those of maximum slope (reached at the beginning of each cleavage), and the minimum slope (between cleavages).

The slopes of the linear parts of these curves were then plotted against time, and the results for the same egg are shown in Text-fig. 13. All cleaving eggs went through a similar cycle of events, but the ratio between the maximum and minimum slopes varied for particular eggs between limits shown in Text-fig. 13, in which measurements for an egg showing the largest variation at first cleavage are plotted together with those for another egg which showed the smallest variation. Values for all other eggs (only one other is shown in Text-fig. 13) lay between these extremes. There was no obvious correlation between the value of the maximum to minimum ratio and the degree of pigmentation of the egg.

Qualitatively then, the mechanical properties of the cortex, as revealed in this experiment, vary with time in a similar manner to that adduced from observations of the 'rounding-up' of the egg at cleavage: the cortical layers of the egg show maximum rigidity at the beginning of cleavage. These results are also broadly similar to the results obtained by Mitchison & Swann (1955) for cleavage in sea-urchin.

Using the simple elastic shell model of the egg, a doubling in the value of the ordinate p/δ in Text-fig. 13 (which records a rather similar quantity to that which Swann and Mitchison call stiffness) would indicate a doubling in the value of Young's modulus for the cortex if its thickness were known to remain constant, or if, on the other hand, Young's modulus were known to remain constant an increase in thickness of 26% would be indicated. The ratio of the maximum value for p/δ at first cleavage to the minimum value after it, is 3·3 for the egg with the greatest variation and 2·35 for the egg showing least variation. If we assume that during cleavage no significant change in Young's modulus for the cortical material takes place, and that no synthesis in the total quantity of cortical material takes place either, but that the existing cortical material is merely distributed by an unspecified process over a larger surface, then taking the values for the eggs showing minimum variation we calculate that the additional surface which could be produced at first cleavage would be 33%, which is in agreement with our previous estimate for the new surface required.



Text-fig. 13. Graphs showing the variation with time during cleavage of the ratios p/δ, measured from the linear portion of curves of p against δ, for eggs at room temperature. The ordinate is proportional to the flexural rigidity of the surface layers. Egg 13 was the egg which showed the greatest degree of variation during the cleavage cycle. Egg 22 showed the least variation.

Measurements on the elastic properties of the cortex give similar values during similar periods of time with respect to the cleavage cycle, no matter whether they are made on pigmented cortex near the furrow, on surface opposite the spindle poles, or on animal or vegetal surfaces. Moreover, when a measurement is made between 20 and 30 min. after the beginning of first cleavage, on the new unpigmented cortex in the furrow region on the animal surface, then here also the value is similar to measurements made elsewhere at the same stage (i.e. the value is slightly above the minimum value). This clearly means that the unpigmented cortex is not the original cortex which has greatly expanded in surface area, for if that were so its small thickness would make it more easily deformable. At the time when the new cortex, having moved out from the furrow region, appears on the surface, its rigidity is as great as the remainder of the cortex.

If, however, the micropipette is deliberately thrust further into the forming furrow, pushing the daughter blastomeres aside in the process, a measurement may be made on surface which gives about half the usual value. This surface is probably either in process of formation or has been damaged in the process of getting the micropipette into position, for at the end of the measurement the pipette cannot be withdrawn easily away to leave an undamaged surface as is normal. In this case the surface adheres to the tip of the micropipette, and long strands of gel stretch between egg and micropipette as the latter is drawn away.

When the furrow begins to move across the animal surface at the beginning of first or second cleavage, the micropipette may be placed for a measurement at the side of the egg in a position which is estimated to be in line with the future course of the furrow. Then an almost hemispherical deformation may be maintained by applying the same pressure reduction which would be required for the micropipette on any other part of the surface at the same time. When this was done, the 'dipping-in' of the furrow eventually occurred quite suddenly to give a sharp cusp in the deformed surface. The shape of the surface then held within the micropipette was consistent with the idea that the initial 'dipping-in' of the furrow is caused by forces (or their resultant) acting towards the interior of the egg from points of application along the line of the forming furrow. One could, of course, give an explanation for this particular result by invoking the theory of Lewis (1939) and Marsland (1939, 1951) but if the furrowing is caused by a contraction of a cortical zone of gelated cytoplasm, then it can be estimated from the shape of the cusp that the zone must be less than 10μ wide.

Using an estimate of 2μ for the thickness of the cortical layer, and putting Poisson's ratio at 0·4, we have calculated (using a formula for the deformation of a clamped end-plate of a cylinder under pressure quoted by Timoshenko, 1940), that the Young's modulus of the cortical material of an egg before cleavage is $1{\cdot}5 \times 10^5$ dynes/cm.^{2}. This value assumes that our deduction that no slip occurs for points on the linear part of the curves of p against δ is correct. It also assumes that the gel is isotropic, which is unlikely if the structures involved here are at all similar to those described by Mitchison (1952). It is, moreover, probable that the inner wall of the gel cortical layer has no definite boundary. There might be a gradual change to the more sol-like properties of the fluid cytoplasm in the interior of the egg, which would make estimates of the thickness of the gel layer unreliable. For these reasons this value for Young's modulus should be regarded as giving the order of magnitude only, and the measurements described in this section are much more reliable when interpreted in terms of changes in elastic properties or dimensions, than when used to evaluate the precise values of the constants involved. It would appear that the values for the Young's modulus of the cortical layer of sea-urchin egg calculated by Mitchison & Swann (1954*b*, 1955), using model experiments, also involve the assumption that the gel is isotropic like rubber, and in addition it was assumed that the physical conditions under which the measurements with the sea-urchin eggs were performed were exactly those of the model experiments with the rubber balls (e.g. the same degree of slipping over the surface of the pipette occurred in both cases).

An additional series of measurements was made upon cleaving eggs whose cortex was repeatedly punctured with a fine needle so that a little yolk was lost each time. Provided that the egg was not damaged so that it failed to cleave and subsequently degenerated, the measurements which were made under these conditions were those which might have been made upon an undamaged egg. There is here no evidence for any internal turgor within the egg, but it should be remembered, on the other hand, that the newt egg does give an efficient healing reaction to small punctures. It is interesting to note that Swann & Mitchison calculated that the turgor within a sea-urchin egg is either very small or absent, using measurements made using the micropipette on eggs in hypertonic sea water (Mitchison & Swann, 1954*b*, 1955). The egg of the newt is much less permeable to small ions and molecules than the sea-urchin egg, and similar methods could not be interpreted so as to give an estimate of internal turgor. In the newt, too, the thickness and Young's modulus for the cortical layer are sufficient to maintain the shape of the egg, without there being an internal pressure within the egg. There will of course be at least a pressure difference of 15 dynes/cm.^{2} over the cortical layer at the vegetative pole of the newt egg due to the specific gravity of the egg, but such a pressure is negligibly small for most considerations.

Finally some measurements were made with a micropipette whose cross-section was an ellipse with a 2·3 : 1 ratio between the major and minor axes. Measurements were made with the long axis of the pipette arranged latitudinally and then longitudinally on the side of the egg, but similar values were obtained in both cases. We could by this method, admittedly rather a rough one, detect no anisotropy in the elastic properties of the egg for elongations in directions within the plane of the cortical surface being deformed.

CHANGES IN SUBCORTICAL MORPHOLOGY DURING CLEAVAGE

Eggs were allowed to develop within the vitelline membrane until first cleavage, and after the stage reached by the cleavage furrow had been noted they were fixed in freshly prepared Smith's fixative for 24 hr.* Serial sections were subsequently cut at 7μ, with the eggs orientated in such a way that the cutting edge of the knife was always normal to the plane of the furrow. Permanent preparations were made for examination under the light microscope after staining with eosin and Delafield's haematoxylin.

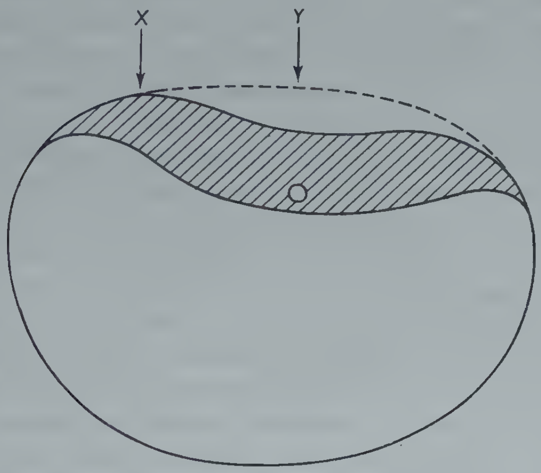
---

* Smith's fixative: 0·5 g. potassium bichromate, 87·5 ml. water, 2·5 ml. 40 % formalin, 10·0 ml. glacial acetic acid.

In the case of almost every egg there were clear signs of changes occurring in the subcortical cytoplasm in positions below and ahead of the furrow, whose progress could be seen on the surface of the egg. The modified cytoplasm lay only in the plane of the cleavage furrow, midway between the daughter nuclei produced by the mitosis and perpendicular to a line joining the two nuclei. The modifications in the appearance of the cytoplasm which lay ahead of the course of the furrow (Pl. 2, figs. 1, 2 and 4–8) consisted first in a displacement of yolk granules, which in a section produced a line containing fewer or no yolk granules; secondly, in local accumulations of small pigment granules along this same line; and thirdly, in fibres which could be seen to lie across and perpendicular to the plane of the modified cytoplasm.

The displaced yolk was always a feature by which the modified cytoplasm ahead of the furrow could be recognized. Accumulations of pigment, in some particular eggs, outlined the future furrow very clearly, but sometimes the pigment granules beneath the egg surface were rather few, just as the total amount of pigmentation on the animal surface also varies widely between different eggs. When an egg was fixed at a time when cleavage could first be recognized on the animal surface, the fibres were most obvious (P. 2, fig. 1). In the Canada balsam of the mounting medium they also showed positive birefringence. These fibres may be the remnants of the spindle, but they could be recognized in regions slightly to the vegetal side of the nuclei and they extend right up to the animal surface (a distance of about 220 μ from a line joining the two nuclei to the animal surface), which would seem to be rather farther towards the animal surface than is usually regarded as normal for a telophase spindle. Spindle remnants can be recognized in the cytoplasm at this stage, however, particularly near the nuclei themselves. They are not found, however, in the yolk immediately on either side of the modified region. The longest fibres which extend across the modified region of displaced yolk are about 25 μ long, when the total distance between the daughter nuclei is about 450 μ. If the fibres in the region of modified cytoplasm are spindle fibre remnants, it is clear that they have been preserved in this region, whereas to either side they have already degenerated. The wide extent of the region in which fibres are found in the plane of modified cytoplasm leads one to infer that this preservation began when the spindle was at anaphase, for at that time the greatest cross-section through the middle of a spindle is far larger than its value in late telophase. Such a preservation could be understood if the modification in the cytoplasm were interpreted as the formation of a gel layer in line with the position of the future furrow. If the gel layer began to form at anaphase in the plane of the greatest cross-section of the spindle, that is midway between the daughter nuclei, then it is easy to understand the displacement of yolk as the layer of gel thickened, and any spindle fibres would be preserved in the gel, for there they would not be subject to appreciable Brownian motion and would be protected from any streaming movements which might take place in the more fluid cytoplasm. For these reasons, and those to be described later arising from the examination of cleaving eggs after centrifugation, we have interpreted the observation of modified cytoplasm as due to the formation of gel inside the egg in the path of the future furrow.

From serial sections of a particular egg, fixed when the furrow had begun to move across the animal surface, the extent of the 'dipping-in' of the animal surface along the line of the furrow was measured, and also the depth to which the cytoplasmic modification ahead of the furrow had extended was measured for each section. These results were used to construct a diagram of a section through the middle of the egg in the plane of the cleavage furrow itself, upon which both the 'dipping-in' and the extent of the observed cytoplasmic modifications were recorded (Text-fig. 14). It will be seen that whereas there are always these signs of gel formation below the course of the furrow which would be observable from outside the egg, there are regions (shown on one side of this particular egg) where the gel layer is observed to extend below the surface where no 'dipping-in' of the surface has yet occurred. A section in such a region is shown in Pl. 2, fig. 2. Although there is almost no depression of the animal surface here, for the furrow has not yet reached this part of the egg, yet the line of displaced yolk extending 330μ into the egg is obvious. A line of subcortical pigment granules can also be seen in the modified region (it is clearest about half-way down) with a layer showing displaced yolk $\sim 4\mu$ thick on either side, so that the gel layer appears double. It is important to note that the pigmented cortex at this stage (after the first 10 min. of cleavage) forms an unbroken layer on the animal surface of the egg across the top of the gel layer which extends into the egg along the line of the future furrow. This observation, and the fact that the surface cortical pigment was not observed to move towards and into the line of the future or early furrow when the animal surface of the cleaving egg was



Text-fig. 14. Diagram of a section through an egg in the plane of the first cleavage furrow, about 8–10 min. after the beginning of first cleavage. The area between the dotted line and the top surface of the egg indicates the extent of 'dipping-in' in the furrow region. The shaded area indicates the extent of the sheet of modified cytoplasm ahead and beneath the externally visible furrow. See also Pl. 2, figs. 1 and 2.

filmed, would seem to rule out any interpretation which assumes that the modified subfurrow region is caused by an expansion or growth of the animal cortex into the line of the furrow. It will be recalled that the movement actually observed was in the opposite direction.

Unpigmented cortex on the animal surface at each side of the furrow, was seen in later sections (Pl. 2, fig. 3) from eggs fixed 20–35 min. after the beginning of cleavage. The implication is that the new unpigmented cortex is synthesized in the region where cytoplasmic modification was observed, and that during mid-cleavage this moves out temporarily on to the animal surface where it was seen in the films of cleavage.

Another group of eggs in first cleavage was centrifuged at between 200 and 400 g. for periods between 5 and 11 min., using a sucrose sugar gradient to support the eggs, which were within their vitelline membranes. The eggs were fixed in Smith's fixative immediately after centrifugation and slides of stained serial sections cut perpendicular to the furrow were prepared as before.

The eggs were not unduly distorted or broken by this rather mild centrifugation. However, some stratification resulted near the animal surfaces of the eggs. In well-pigmented eggs not only is there pigment in the surface layers of the cortex but there is a considerable accumulation of pigment immediately below it so that the total surface layer of pigment which can be seen in a section may be fully 5μ thick. The centrifugation caused displacement of a large part of this pigment from the immediately subcortical layers below the animal surface towards the centrifugal pole, but the surface pigment in the cortex was not moved (Pl. 2, fig. 7). In the furrow region, however, no stratification was ever seen, and the subcortical pigment was not displaced at all. This may have been due to the rigidity of the subcortical gel beneath the furrow, but the furrow in the case of most eggs fixed after centrifugation was rather too far advanced to make this conclusion certain. (Earlier stages of cleavage are difficult to examine after centrifugation because of the time which is spent in the centrifuge.) Nearly all the sectioned eggs fixed after centrifugation showed clear signs of modified cytoplasm extending into the interior of the egg ahead of the cleavage furrow, and in several cases this was bent or distorted, presumably as a result of the centrifugation (Pl. 2, fig. 8). That the modified cytoplasm can be bent about without being destroyed seems clear confirmation of its rigid gel properties. In several instances, moreover, the pigmentation in the modified region formed two lines of granules at a separation of about 4μ, and this suggests that the new layer of gel may already itself be double.

THE INTERPLAY OF THE VARIOUS FACTORS

It is possible to link together all the known facts about cleavage in the newt by forming a comprehensive theory from the deductions we have already made:

From a time which may vary for particular eggs from 20 to about 50 min. before the first cleavage furrow begins at the animal pole of the egg, the cortical layer begins to become a more rigid structure. This causes the egg to 'round-up' and assume a more spherical shape as it becomes less easily flattened under gravity, and the surface also becomes less easily deformable in experiments with the micropipette. By the beginning of cleavage this change has reached its maximum value, and the structural layer of cortical gel may be assumed then to have increased in thickness by between 30 and 48% if we assume no change in its elastic moduli, although an increase in the value for Young's modulus (of between 130 and 230%) would also give the same result.

Beginning at the time of late anaphase of the nuclear division, new cortex begins to form inside the egg in a plane defined by the mid-points of the spindle fibres (i.e. in the plane of the future furrow). The new cortical gel grows in extent from the animal region, where the spindle is located, and extends gradually into the interior of the egg, towards the side of the egg, and towards the vegetative pole. Signs of the new cortical gel are seen in the sectioned eggs, and there are indications that the gel layer being formed is double. When the new gel has increased in area to about one-sixth of the cross-sectional area of the egg, extending downwards from the animal polar surface, it contracts. Now the upper edge of this area of newly formed gel is an arc which follows the line of the animal surface, and it is joined to the animal surface along this line, so that a contraction of new subcortical gel results in a contraction in the length of its upper boundary also. This contraction was recorded in the analysis of film sequences (Text-fig. 7); it manifests itself in the temporary surface wrinkles on the coat transverse to the first signs of the furrow; it also causes the movement of surface pigment towards the first signs of the furrow from regions in line with the future course of the furrow at the side of the egg, where it is free to move because the new subcortical gel has not yet formed beneath it. The area of subcortical gel continues to increase by growth at its lower edge and the subsequent contraction which occurs later is considered as a phase in the formation of new cortex inside the egg. When its area is about two-thirds that of the total section of the egg, the contraction merely causes a 'dipping-in' as the externally visible furrow reaches to the sides of the egg. At about this time the new unpigmented cortex immediately below the animal surface is fully formed and is a double layer. The coat along the top of the furrow then parts in two, and as the daughter blastomeres tend to fall apart under gravity, the furrows open out to expose new unpigmented cortex on the egg surface in the furrow region, the junction between the old pigmented cortex and the new unpigmented cortex being quite obvious. This is about 20 min. after the beginning of first cleavage. The subcortical gel layer finally grows to meet the vegetal surface of the egg and its subsequent contraction helps to pull the new unpigmented cortex on the animal surface back into the furrow region. When the double layer of new cortex, formed entirely within the egg, is complete in the vegetal region also, the old surface cortex parts, and since it has already joined with the new, the two daughter blastomeres are then fully separate.

The movements and expansions of the pigmented cortex on the surface of the egg, which were described in an earlier section, are always those which might be expected of an elastic surface shell while the new subcortical gel is being synthesized in the manner described above. Its final surface area is unchanged. The additional surface area produced by cleavage is entirely supplied by new unpigmented cortex synthesized inside the egg and forming those boundary surfaces by which the blastomeres remain in contact after cleavage (and with the slight increase in surface area of a few per cent required on the surface which is externally visible, also supplied by unpigmented cortex visible after cleavage in the furrow region). All cleavage subsequent to the first takes place similarly. The result is that when a morula and later a blastula is formed, the total surface area of the pigmented cortex still has not increased and it still lies on the outside of the blastula, while the inner and contact surfaces of the cells are of the newer unpigmented cortex. The sum total increase in surface area of all the blastomeres is thus in the form of unpigmented cortex. This is in accord with observation.

The manner of the formation of new cortex in the interior of the egg has not so far been considered. We have deduced that gel is forming ahead of the furrow in a double layer. The decrease in rigidity of the entire surface cortex takes place during precisely the period in which the new sub-cortical gel is forming. Some transfer mechanism is indicated. Simple expansion of the surface of the original cortex does not take place; if it did, the movement of pigment would have been obvious. If simple expansion of the inner part of the pigmented cortex was possible while leaving the surface layers at rest, then the observations made on surface pigment could be met, but in that case our deductions concerning the spindle fibres and the forming subcortical gel are certainly false and we would expect to see no fibres in the new subcortical gel. It seems better to assume that the transfer implies a change from gel to sol at the inner surface of the pigmented cortex during cleavage, and from sol to gel along the lower edge of the new unpigmented cortex being formed ahead of the furrow. Such a mechanism implies analogies between amoeboid movement and cleavage, and also suggests that streaming movements in the more fluid cytoplasm take place. Both these suggestions have been made by previous authors (e.g. Davson, 1951; Marsland, 1950), but it is difficult to see how streaming might be detected in newt. However, streaming movements of the general form suggested by Spek (1918, see Wilson, 1928), and observed by him in other species, would seem to be of a kind which would support the above hypothesis, since the flow is round and under the surface of the blastomeres and then towards the furrow region.

That the cortical gel is first synthesized before cleavage, as an additional layer beneath the pigmented cortex already existing, and then transferred to the required region during cleavage, is in line with a number of recent cytological discoveries in which the period before mitosis is revealed as a time of active chemical synthesis, and the mitotic period itself is one of active movement and transport with the minimum synthesis of new materials.

DISCUSSION AND COMPARISON WITH CLEAVAGES IN OTHER FORMS

The most obvious difference between the cleavage process as described here for the newt, and as observed by other authors for the much-studied echinoderms, is that very large surface expansions of the original cortex apparently take place in the latter.

After the hyaline layer has been removed, the first part of the cleavage process in sea-urchin results in the formation of two spherical daughter blastomeres apparently entirely covered by the expanded former cortex. In species with thin hyaline membranes (e.g. *Astricylpeus manni*) the cortex behaves similarly in the natural state; in species with thicker hyaline membranes (*Mesipulus globulus*) the movement of the cortex is inhibited in all except the furrow region (Dan *et al.*, 1938). Measurements of this expansion process were usually made between kaolin particles along the circumference of the largest optical section of the egg, after removal of the hyaline layer (Dan, Yanagita & Sugiyama, 1937; Dan *et al.* 1938; Dan & Dan, 1940). The measurements show linear increases ranging in value from about 10 % opposite the spindle poles to about 100 % in the furrow region, and with an average value which can be computed graphically to be about 40 %. Yet the total increase in surface area possible is only 26 %. The measurements of large linear expansions along the largest optical section of the egg then clearly imply that linear contractions are meanwhile taking place in directions at right angles, that is for directions along the surface around the axis of rotation of the egg. Linear measurements in such directions have never been reported to our knowledge. It is calculated that for linear measurements along the surface in such directions an average contraction of about 10 % should be found; no difference between linear measurements made in different directions would be expected in the polar region; for subpolar and subfurrow regions linear increases in directions round the axis of rotation would be expected to be appreciably less than for directions along the largest optical section, while in the furrow region itself there must, for directions round the axis of rotation, be not linear expansion, but linear contractions up to about 40 %. Such a scheme, deduced from the measurements of Dan and his co-workers, allows the net surface area increase in the first phase of cleavage in echinoderms to be 26 % with the hyalin layer absent. Whereas much attention has been given to the expansions in formulating theories of cleavage (e.g. Mitchison, 1952), almost no attention seems to have been drawn to the contractions before the recent paper of Dan & Ono (1954), where areal measurements, made for particular regions of surface, were reported, and an areal contraction was found for the furrow region. Moreover, their figure 12 clearly shows linear contraction along the forming furrow.

A study of the work of Dan (1954*a*, *b*) upon echinoderms which have pigment granules uniformly distributed round the surface of the egg before first cleavage, leads one to infer that the net result of cleavage in newt and sea-urchin is very similar. Here also the pigmented surface remains on the outside while new unpigmented cortex forms the boundary by which the blastomeres remain in contact after a division inside the hyaline layer. In the subsequent blastula stage the pigment is still on the outside surface of the larva (Motomura, 1935). It is also a point of similarity with the newt that the new unpigmented cortex appears to grow in a region midway between the daughter nuclei, but in cleavages studied after the hyaline layer has been removed this event occurs after the blastomeres have been already separated by the process involving an approximately 26 % expansion of the original cortex. Here the growth of new cortex appears superficially to be a post-cleavage event, but its growth nevertheless causes the surface area of pigmented cortex to return to its initial value as in the newt; and so this process is to be regardedas the second phase of cleavage (Motomura, 1950).

It would appear that whereas the cortical movements during cleavage in echinoderms are much greater, when recorded as percentages, than in the newt, yet they do not differ qualitatively except in the matter of timing. The difference between the formation of new cortex in the interior of the egg, as in the newt, or on the surface after the blastomeres have been separated, as in echinoderms cleaving after the hyaline layer has been removed in calcium-free sea water, is merely a matter of the temporal delay of this process in the echinoderms. As regards surface movements, the similarity between newts and echinoderms would probably be closest when the comparison is made with species such as *Astricylpeus* where the spindle is also towards the animal surface.

The correspondence between the general form of the variation in rigidity of the cortical layers during cleavage for newt and sea-urchin has already been noted. Motomura (1950) examined paraffin sections through eggs during cleavage in several species of sea-urchin, and he observed a more lightly staining zone ahead of the cleavage furrow at the beginning of cleavage. There was also an accumulation of granules with specific staining properties round vacuoles in this region, and these structures appeared to Motomura to play a role in the subsequent formation of new surface in the furrow. As described above, we have also found special features in the cytoplasm preceding the appearance of a definite furrow. Thus there is very considerable correspondence in observation and measurement between studies made upon echinoderm and amphibian eggs during cleavage. There is no corresponding similarity, however, between the hypotheses which have been propounded to account for the results obtained.

The ideas which have been suggested here to account for cleavage in the newt do, however, provide a hypothesis for cleavage in echinoderms also. In the first place it is worth noting that for echinoderms as well as newts the various movements of the surface cortex and the linear and areal expansions and contractions are always least at the polar and greatest in the furrow region, with intermediate values in the subfurrow and subpolar regions. The most obvious explanation for this is that the cause of the movement lies in the furrow region. The region of subcortical cytoplasmic modification ahead of the forming furrow was regarded by Motomura (1950) as a region of cytoplasmic weakness, into which the original cortex would presumably expand. He supported this idea by the observation that this region contracted most when the eggs just prior to cleavage were placed in hypertonic sea water. This observation could also have been interpreted by supposing that the dehydration of the region produced by the hypertonic sea water only assisted in the contraction of the modified and gelating cytoplasm which occurs during the first phase of normal cleavage. It is possible that the wave of decreasing birefringence which Swann (1951*b*, 1952) observed to spread from near the centre of each aster outwards to the cell surface, reaching the cortex first opposite the spindle poles, was associated with a progressive solution of the inner surface of the original cortex also beginning opposite the spindle poles, and this would allow a freer expansion of the surface cortex and also would provide material for the gel which is forming and contracting beneath the furrow. An alternative explanation would be in terms of 'active expansion' of the original cortex involving changes in the folding of protein chains as outlined by Mitchison (1952). This would not, in our view, account for the initial 'dipping-in' of the new furrow, but the difficulty might be circumvented by postulating 'active contractions' in the furrow region itself. In either case the results of the changes in rigidity measured with the micropipette at various points on the cortex of the sea-urchin egg during cleavage will be of interest both during the phase when the original cortex is expanding and subsequently when it is, in our view, being returned to its original area by the formation of new surface in the furrow region. One of the points which future work must settle will be whether the greater percentage linear and areal changes observed in the original cortex of the sea-urchin during cleavage, are primarily due to the cortex of that form being either thinner or possessing smaller elastic moduli than in the newt, or whether the active expansions and contractions of the original cortex are really more important and greater in sea-urchin. It will have been noted that our explanation of cleavage in the newt does not involve 'active expansion' of the cortex.

An objection which is frequently raised against a hypothesis of cleavage which involves contraction in the furrow region is that, when applied to the cleavage of the sea-urchin in a calcium-free medium with the hyalin layer removed, the contracting region must apparently contract away to nothing, for at the end of the first phase of cleavage the daughter blastomeres are connected by the thinnest protoplasmic stalk. In the newt this difficulty does not exist, for the contraction of the original cortex, which we observed on the surface of the egg in the furrow region and parallel to the furrow, is not so great. Furthermore, throughout cleavage in the newt, new gel is being progressively added to form new cortex inside the egg ahead of the newly formed cortex which is contracting. In the sea-urchin, as well as in the newt, it is suggested that before the contraction phase is completed the contracting gel is already double, so that when, in sea-urchins cleaving in a calcium-free medium, the contraction phase finally reaches its fullest extent the daughter blastomeres are seen to be separate. It is noteworthy that both Dan and Motomura have reported that, at this particular stage, the extra granular zone on both daughter blastomeres is noticeably thicker round the base of the stalk, and that the coloured granules (when present) accumulate here also, and both observations suggest a contracted state.

To complete the analogy with the newt, we accept Motomura's conclusion (Motomura, 1950) that new unpigmented surface is formed in this region, where the characteristically staining granules were found to be concentrated. The new unpigmented surface, as in the newt, accounts for the whole of the increase in surface area finally produced by cleavage, and the growth of the new surface returns the pigmented original cortex to its original area, as the areal measurements of Dan (1954*a*) clearly show. Dan (1954*a*), however, apparently believes that the new unpigmented surface is merely the original surface locally stretched, but this could easily be tested by making rigidity measurements upon it with a micropipette; since the deformability of the surface layer depends inversely upon the third power of its thickness, if Dan is correct it should be very easily deformable in comparison with the pigmented cortex at the same stage. It will be recalled that in the newt no difference in elastic properties of the pigmented and the new unpigmented cortex could be detected.

For cleavages in the higher plants the idea of the formation of a double cell surface, growing from the position of the spindle remnant outwards to join with the original cell surface, seems to fit the published evidence with which we are familiar. It seems possible, therefore, that cleavages in plants may be understood without having to introduce new ideas, but considerably more work remains to be done before one could discuss plant cleavage in any detail. We have no reason to doubt at the moment, however, that cleavages in all plant and animal forms may not take place by processes which are fundamentally similar, in which the points of difference are points of detail due to differences in the relative importance of the many factors involved.

SUMMARY

The early cleavages in eggs of *Triturus alpestris* have been studied.

Ciné-film technique was used to record changes in the shape of the egg and movements of the surface pigment from which measurements of linear and areal changes were made. Local vital staining was also employed. There was no significant net change in the area of pigmented cortex during cleavage.

Before cleavage the egg resembles a viscous liquid drop whose shape is maintained by a uniform elastic shell of Young's Modulus $\sim 1\cdot5 \times 10^5$ dynes/cm.^{2} and thickness about 2μ. The egg assumes a more nearly spherical shape immediately before cleavage when the flexural rigidity of the surface layers increases. The flexural rigidity of the cortical layers was found to be maximal at the beginning of cleavage and minimal midway between cleavages. This variation is similar to that previously recorded for cleavage in sea-urchin eggs by Mitchison & Swann (1955), using a similar method. At any particular stage with respect to the cleavage cycle no variation was found in the rigidity at different points on the egg surface.

Serial sections show cytoplasmic modification below and ahead of the forming furrow. It was concluded that the new unpigmented cortex, by which the daughter blastomeres remain in contact after cleavage, is first formed as a sheet of gel (which in later stages can be seen to be a double layer) which grows downwards by a process involving gelation at its lower edge, through the cytoplasm from the animal toward the vegetal surface. The gel layer is assumed to contract immediately after its formation, and in this way to produce 'dipping in' of the new furrow and all the observed surface movements. These ideas have been developed to form a detailed theory of cleavage in the newt, and suggest a common basis for the consideration of cell division in echinoderm eggs, plants and other forms, on the basis that the necessary increase in surface area is achieved by the formation of new cortex rather than by the expansion of the original cell membrane.

The authors wish to thank Prof. M. M. Swann and Dr J. M. Mitchison, both of the Zoology department, Edinburgh University, for helpful discussion during the course of this work. The ciné films used in the course of this study were made by Mr E. Lucey of this department. Measurements of the rate of furrowing and much of the work using a local vital staining method was performed by Miss H. Yates of this department. Dr E. Deuchar, now at University College, London, prepared some of the earlier sectioned material, and we thank Dr M. Fischberg, Oxford University, for a suggestion concerning spindle fibres. The work received the financial support of the Agricultural Research Council.

REFERENCES

CARLSON, J. G. (1952). Microdissection studies of the dividing neuroblast of the grasshopper *Chorthippus viridifasciata* (De Geer). *Chromosoma*, **5**, 199–220.

CHAMBERS, R. (1938). Structural and kinetic aspects of cell division. *J. Cell. Comp. Physiol.* **12**, 149–65.

DAN, K. (1943). Behaviour of the cell surface during cleavage. VI. On the mechanism of cell division. *J. Fac. Sci. Tokyo Univ.*, Ser. IV, **6**, 323–68.

DAN, K. (1954*a*). Further study on the formation of 'new' membrane in eggs of the sea urchin *Hemicentrotus* (*Strongylocentrotus*) *pulcherrimus*. *Embryologia*, **2**, 99–144.

DAN, K. (1954*b*). The cortical movement in *Arbacia punctulata* eggs through cleavage cycles. *Embryologia*, **2**, 115–121.

DAN, K. & DAN, J. C. (1940). Behaviour of the cell surface during cleavage. III. On the formation of the new surface in the eggs of *Hemicentrotus* (*Strongylocentrotus*) *pulcherrimus*. *Biol. Bull. Woods Hole*, **78**, 486–501.

DAN, K., DAN, J. C. & YANAGITA, T. (1938). Behaviour of the cell surface during cleavage. II. *Cytologia*, **8**, 521–31.

DAN, K. & ONO, T. (1954). A method of computation of the surface area of the cell. *Embryologia*, **2**, 87–98.

DAN, K., YANAGITA, T. & SUGIYAMA, M. (1937). Behaviour of the cell surface during cleavage. I. *Protoplasm*, **28**, 66–81.

DAVIES, H. G., WILKINS, M. H. F., CHAYEN, J., & LA COUR, L. F. (1954). The use of the interference microscope to determine dry mass in living cells and as a quantitative cytochemical method. *Quart. J. Micro. Sci.* **95**, 271–304.

DAVSON, H. (1951). *A Textbook of General Physiology*. London: J. and A. Churchill Ltd.

FARR, C. H. (1918). Cell-division by furrowing in Magnolia. *Amer. J. Bot.* **5**, 379–95.

FUJII, K. & YASUI, K. (1954). On the mechanism of nuclear division and chromosome arrangement (A symposium). III. Studies on nuclear division *in vivo*. B. Meiotic processes from the first prometaphase to the end of the second division of the PMC. *Cytologia*, **19**, 54–76.

GRAY, J. (1924). The mechanism of cell division. I. The forces which control the form and cleavage of the eggs of *Echinus esculentus*. *Proc. Camb. Phil. Soc. Biol. Sci.* **1**, 164–88.

HARVEY, E. N. & FANKHAUSER, G. (1933). The tension at the surface of the eggs of the salamander *Triturus* (*Diemyctylus*) *viridescens*. *J. Cell. Comp. Physiol.* **3**, 463–75.

HOLTFRETER, J. (1943). Properties and functions of the surface coat in amphibian embryos. *J. exp. Zool.* **93**, 251–319.

LEWIS, W. H. (1939). The role of a superficial plasmagel layer in changes of form, locomotion and division of cells in tissue cultures. *Arch. exp. Zellforsch. Gewebzücht.* **23**, 1–7.

MARSLAND, D. A. (1939). The mechanism of cell division. Hydrostatic pressure effects upon dividing egg cells. *J. Cell. Comp. Physiol.* **13**, 15–22.

MARSLAND, D. A. (1950). The mechanisms of cell division; temperature-pressure experiments on the cleaving eggs of *Arbacia punctulata*. *J. Cell. Comp. Physiol.* **36**, 205–27.

MARSLAND, D. A. (1951). The action of hydrostatic pressure on cell division. *Ann. N.Y. Acad. Sci.* **51**, 1237–35.

MITCHISON, J. M. (1952). Cell membranes and cell division. *Symp. Soc. Exp. Biol.* **6**, 105–27.

MITCHISON, J. M. (1953). Microdissection studies on sea-urchin eggs at cleavage. *J. Exp. Biol.* **30**, 515–24.

MITCHISON, J. M. & SWANN, M. M. (1952). Optical changes in the membranes of sea urchin eggs at fertilization, mitosis and cleavage. *J. Exp. Biol.* **29**, 357–62.

MITCHISON, J. M. & SWANN, M. M. (1954*a*). The mechanical properties of the cell surface. I. The cell elastimeter. *J. Exp. Biol.* **31**, 443–60.

MITCHISON, J. M. & SWANN, M. M. (1954*b*). The mechanical properties of the cell surface. II. The unfertilized sea-urchin egg. *J. Exp. Biol.* **31**, 461–72.

MITCHISON, J. M. & SWANN, M. M. (1955). The mechanical properties of the cell surface. III. The sea-urchin egg from fertilization to cleavage. *J. Exp. Biol.* **32**, 734–50.

MOTOMURA, I. (1935). Determination of the embryonic axis in the eggs of Amphibia and Echinoderms. *Sci. Rep. Tôkohu. Univ.*, Ser. IV, **10**, 211–45.

MOTUMURA, I. (1950). Studies of cleavage. V. The role of vacuoles in the cleavage-plane formation in sea-urchin eggs. *Sci. Rep. Tôhoku Univ.*, Ser. IV, **18**, 255–61.

NELSEN, O. E. (1952). *Comparative Embryology of Vertebrates.* London: Constable and Co. Ltd.

SCHECHTMANN, A. M. (1937). Localised cortical growth as the immediate cause of cell division. *Science*, **85**, 222–223.

SCHRADER, S. H. (1948). Cytology of Coccids (Coccoidea-Homoptera). *Advances in Genetics*, **2**, 127–203.

SPEK, J. (1918). Oberflächenspannungdifferenzen als eine Ursache der Zellteilung. *Arch. EntwMech. Org.* 44, 5–113.

SWANN, M. M. (1951*a*). Protoplasmic structure and mitosis. I. The birefringence of the metaphase spindle and asters of the living sea-urchin egg. *J. Exp. Biol.* **28**, 417–33.

SWANN, M. M. (1951*b*). Protoplasmic structure and mitosis. II. The nature and cause of the birefringence changes in sea-urchin egg at anaphase. *J. Exp. Biol.* **28**, 434–44.

SWANN, M. M. (1952). The nucleus in fertilization, motosis and cell division. *Symp. Soc. Exp. Biol.* **6**, 98–104.

SWANN, M. M. & MITCHISON, J. M. (1953). Cleavage of sea-urchin eggs in colchicine. *J. Exp. Biol.* **30**, 506–14.

TIMOSHENKO, S. (1940). *Theory of Plates and Shells.* New York: McGraw Hill.

TYLER, A. (1942). Developmental processes and energetics. *Quart. Rev. Biol.* **17**, 197–212.

WADDINGTON, C. H. (1952). Preliminary observations on the mechanism of cleavage in the amphibian egg. *J. Exp. Biol.* **29**, 484–9.

WILSON, E. B. (1928). *The Cell in Development and Heredity.* New York: MacMillan Co.

EXPLANATION OF PLATES

PLATE 1

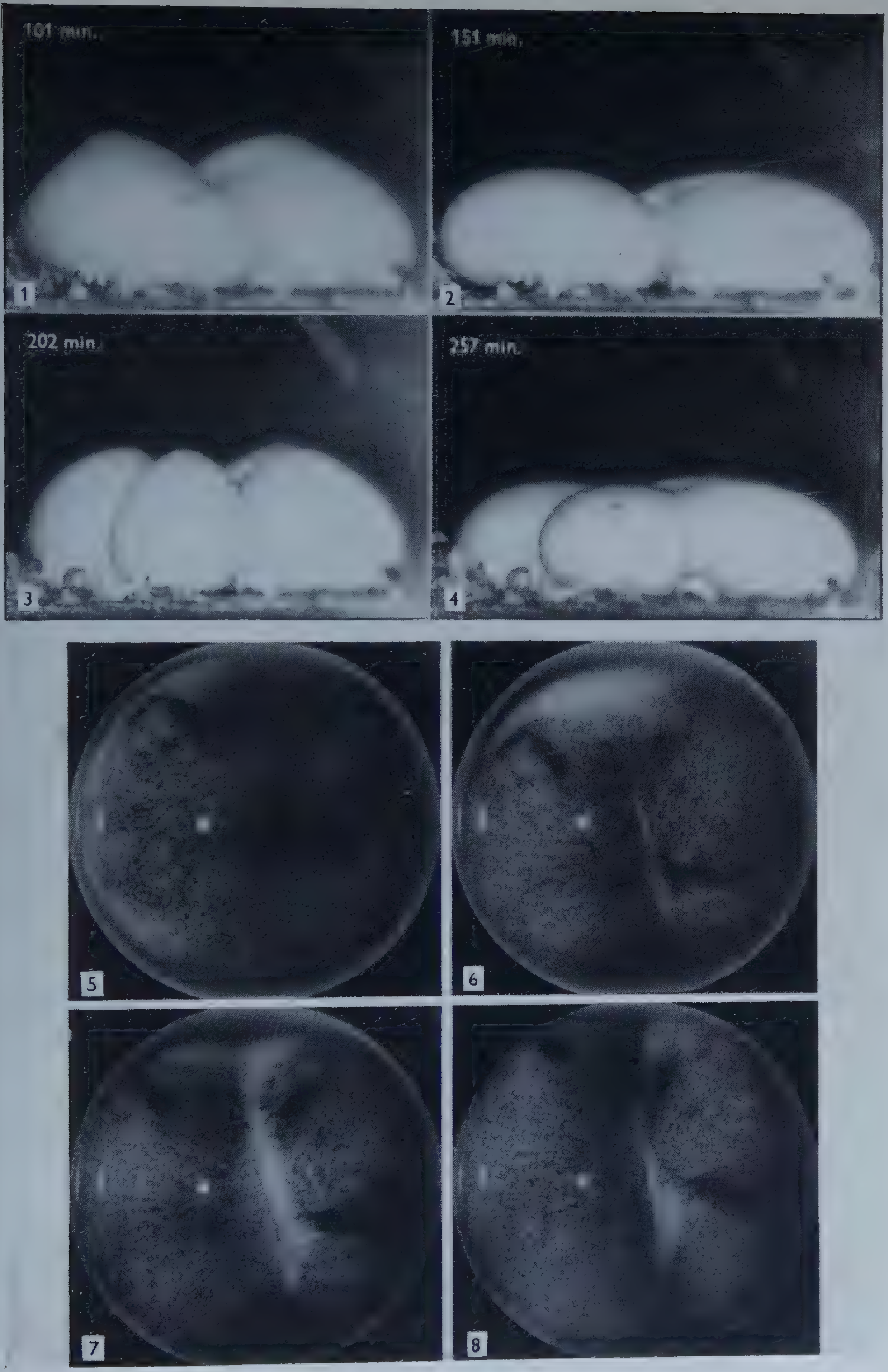
Figs. 1–4. Illustrations from a film sequence of an egg cleaving on a flat surface after the vitelline membrane had been removed. Fig. 1, a 'rounded-up' position of the egg at first cleavage. Fig. 2, a 'relaxed' position of the egg between first and second cleavage. Fig. 3, a 'rounded-up' position of the egg at second cleavage; note that one of the blastomeres is of greater height than breadth. Fig. 4, the subsequent 'relaxed' position of the egg between second and third cleavage.

Figs. 5–8. Egg viewed from above the animal pole during first cleavage within the vitelline membrane, from a film sequence to show pigment movement. A slight rolling movement of the egg about the vegetative pole takes place during cleavage. Fig. 5, 3 min. after the first sign of cleavage; contraction and 'dipping-in' is taking place along the line of the new furrow. Fig. 6, 8 min. after the beginning of cleavage; note the first signs of the furrow opening. Fig. 7, 16 min. after the beginning of cleavage; the early furrow has opened out to reveal an area of new white unpigmented cortex. Fig. 8, 40 min. after the beginning of cleavage. The furrow is closing again, although some white surface still remains in the furrow at the animal pole.

PLATE 2

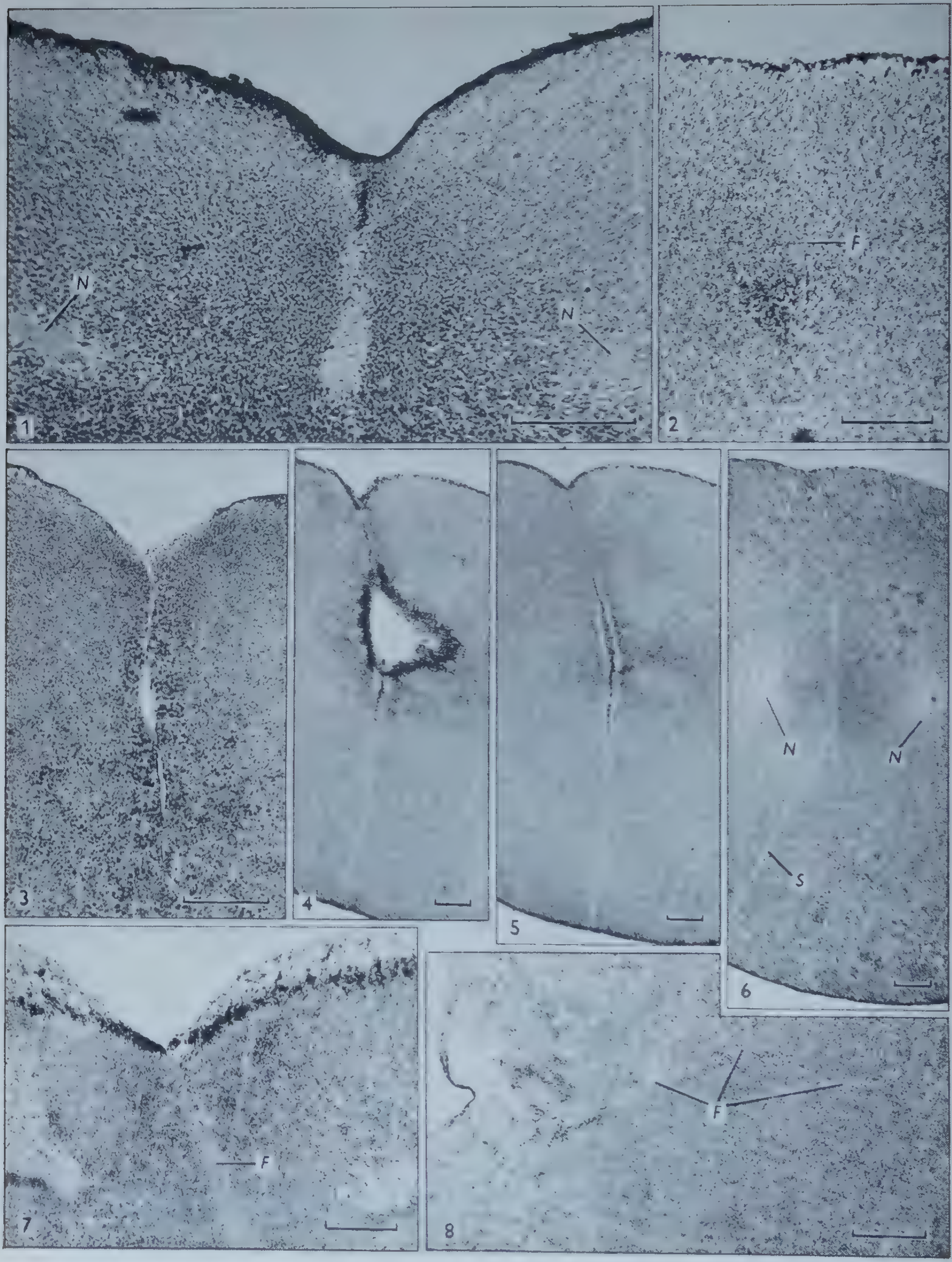
(Microphotographs of sectioned eggs, fixed in Smith's fixative during first cleavage, and stained with eosin and Delafield's haematoxylin. In all cases the drawn scale represents 100 μ.)

Fig. 1. Vertical section cut transverse to the cleavage furrow, through the daughter nuclei *N*, about 8 min. after the first sign of cleavage (see position *Y* of Text-fig. 14). Note the fibres in the area of displaced yolk.



SELMAN AND WADDINGTON—THE MECHANISM OF CELL DIVISION IN THE CLEAVAGE OF THE NEWT'S EGG

(Facing p. 732)



SELMAN AND WADDINGTON—THE MECHANISM OF CELL DIVISION IN THE CLEAVAGE OF THE NEWT'S EGG

Fig. 2. Vertical section cut transverse to the cleavage furrow (8 min. after the first sign of cleavage) in a region where the 'dipping-in' of pigmented surface had not yet taken place (see position *X* of Text-fig. 14). Note the line of pigment granules and displaced yolk along the line of the future furrow *F*.

Fig. 3. Vertical section cut transverse to a fully formed cleavage furrow, from an egg fixed 35 min. after the beginning of cleavage. Note that the egg surface at this stage is unpigmented up to about 100 μ on either side of the furrow.

Fig. 4. Section cut in a plane about 10° from the horizontal and transverse to the furrow, at grazing incidence to the 'dipped-in' region of the furrow at the animal pole. Egg fixed about 8 min. after the first sign of cleavage.

Fig. 5. Section cut 40 μ below that shown in fig. 4, from the same egg. Note that the subcortical signs of the future furrow extend right across the egg. Note the line of pigment granules, and that the lighter staining line of displaced yolk is double.

Fig. 6. Section cut 230 μ below that shown in fig. 5 so as to include the daughter nuclei *N*. Note the line of modified cytoplasm midway between the nuclei. The line *S* is an artifact caused by an imperfection of the knife edge. In no case was an egg sectioned so that such an artifact lay parallel to a line of modified cytoplasm.

Fig. 7. Vertical section cut perpendicular to the line of the cleavage furrow across the animal surface. Egg fixed after centrifugation at 1700 r.p.m. for 5 min. after the first signs of cleavage. Note the displacement of pigment and cytoplasmic stratification to either side of the furrow. Note the line of the future furrow *F*.

Fig. 8. Vertical section cut perpendicular to the line of the cleavage furrow across the animal surface. Egg fixed after centrifugation at 1200 r.p.m. for 11 min., after the first signs of cleavage. Note that the line of the future cleavage furrow *F* has been bent by the centrifugation.

THE MECHANICAL PROPERTIES OF THE CELL SURFACE

III. THE SEA-URCHIN EGG FROM FERTILIZATION TO CLEAVAGE

BY J. M. MITCHISON AND M. M. SWANN
Department of Zoology, University of Edinburgh; Marine Station, Millport; and Stazione Zoologica, Naples

(*Received* 22 *December* 1954)

INTRODUCTION

In earlier papers (Swann, 1952; Mitchison, 1952) we put forward a new conception of the mechanism of cell division—the expanding membrane theory. This postulated an active expansion of the cell membrane or cortex as the immediate cause of cleavage; it implied that the cell membrane must be sufficiently rigid to expand in area without buckling, and that there could be little or no internal pressure in the cell during cleavage. We produced evidence in support of this theory from optical changes (Mitchison & Swann, 1952), from the effect of colchicine (Swann & Mitchison, 1953), and from micro-dissection (Mitchison, 1953). It was clearly important, however, to test a mechanical theory of this kind by measuring the mechanical properties of the cell membrane during cleavage. We therefore developed a method of measuring the properties of the cell membrane with an instrument we have called the 'cell elastimeter', which is described in the first paper of this series (Mitchison & Swann, 1954*a*). The second paper of the series (Mitchison & Swann, 1954*b*) described the application of this method to the unfertilized sea-urchin egg, and showed amongst other things that the cell membrane behaved as a relatively rigid structure of appreciable thickness. In the present paper we shall describe measurements of the stiffness and, indirectly, of the internal pressure of the sea-urchin egg from fertilization to the second interphase. We shall also briefly discuss these measurements in relation to other work, and to our own theory of the mechanism of cleavage. A full consideration of all the points involved will be published elsewhere.

STIFFNESS CHANGES DURING THE FIRST INTERPHASE AND DIVISION

The measurements of stiffness changes in fertilized sea-urchin eggs were made with the cell elastimeter using the experimental technique and methods of calculation described in the first and second paper of this series. The fertilization membranes were removed by passing the eggs through fine-mesh bolting silk about 2 min. after fertilization, and the eggs were measured both in ordinary sea water and in calcium-free artificial sea water, which removes the hyaline layer.

The results from a series of measurements on *Psammechinus miliaris* are given in Table 1. The eggs are grouped by stages of development and the stiffness is the average value for each group. This stiffness is given in dynes/cm.^{2}/μ deformation corrected for the standard condition of a 100μ diameter egg and a 50μ diameter pipette.

A difficulty arises in the case of the eggs which were measured when they were in the process of cleaving. Strictly speaking, neither the model experiments nor the correction process described in the first paper are applicable to the irregular shape of cleaving eggs. We have, however, treated eggs at this stage as though they were spheres of the same diameter as eggs before cleavage (105μ). This is somewhat arbitrary, but no other possible treatment seems to be any better. The effect is to make the corrected stiffness values for eggs in the later stages of cleavage somewhat smaller than the true values. If, on the other hand, these eggs had been treated as spheres of the same diameter as the blastomeres in second interphase (83μ), the stiffness values would have come out greater than the true values and would have been 1·52 times the values given in Table 1.

Table 1. *Stiffness of the membrane during development*, Psammechinus miliaris

(Corrected stiffness in dynes/cm.^{2}/μ deflexion for 100μ diameter egg and 50μ diameter pipette)

Stage	Approximate times after fertilization (min.)	Ordinary sea water			Calcium-free sea water		
		Corrected stiffness	Young's modulus × 10^4 dynes/cm.^{2}	No. of eggs	Corrected stiffness	Young's modulus × 10^4 dynes/cm.^{2}	No. of eggs
Unfertilized	—	8·2	0·91	50	9·3	1·03	10
Early sperm aster	3–15	5·2	0·58	8	4·2	0·47	9
Late sperm aster	16–30	8·4	0·93	12	4·5	0·50	13
Streak	31–40	10·3	1·14	9	3·4	0·38	11
Prophase, metaphase, early anaphase	41–53	12·6	1·40	12	5·4	0·60	20
Mid anaphase	54–55	33·9	3·75	2	20·1	2·24	5
Late anaphase, early cleavage	56–58	61·3	6·81	9	57·0	6·32	8
Mid-cleavage, late cleavage	59–60	39·4	4·36	6	49·0	5·44	5
Interphase	61–	17·7	1·96	6	12·5	1·39	8

Note. (1) Average diameter of uncleaved eggs, 105μ, average diameter of blastomeres of cleaved eggs, 83μ. (2) Pipette diameter, 50μ. (3) The same batch of unfertilized eggs which gave the stiffness of 9·3 in calcium-free sea water, gave a stiffness of 9·5 in ordinary sea water.

The figures for Young's modulus are derived from the corrected stiffness values by the method described in the first paper. It should be emphasized that they are only approximate, and that they assume a membrane thickness of 1·6μ and no internal pressure in the egg.

The stiffness values from Table 1 shown are in graphical form in Fig. 1, where the stiffness for each stage is plotted against the central time for that stage. A dotted line (whose maximum height is uncertain) is put in immediately after fertilization. This is derived from the results given in the next section (p. 738).

The results in ordinary sea water show a sudden rise in stiffness at fertilization followed by a fall, during the early sperm aster stage, to the lowest value reached during development. The stiffness then rises slowly until metaphase, after which it rises rapidly to reach a maximum during late anaphase and the early stages of cleavage. During the later stages of cleavage (when the furrow is cutting through the egg) the stiffness falls again and reaches a value in the second interphase which is more or less constant but is about twice as high as in the first interphase. The stiffness alters in all about twelvefold, the lowest value being in the early sperm aster stage, and the highest value in late anaphase (a change in Young's modulus from $0{\cdot}58 \times 10^4$ to $6{\cdot}81 \times 10^4$ dynes/cm.^{2}).

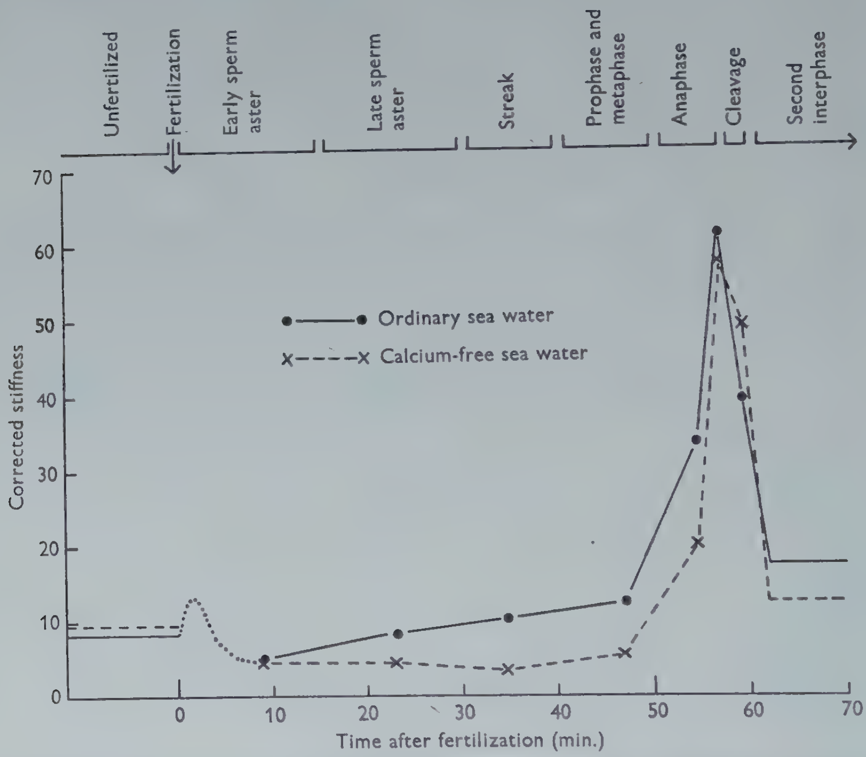


Fig. 1. Stiffness changes during first interphase and division. Eggs of *Psammechinus miliaris*. Corrected stiffness is in dynes/cm.$^2/\mu$ deformation for $100\,\mu$ diameter egg and $50\,\mu$ diameter pipette.

The results in calcium-free sea water show the stiffness changes after the removal of the hyaline layer which appears on the outside of fertilized eggs during the sperm aster stage. There is no significant difference between the values in ordinary and in calcium-free sea water when the hyaline layer is not present, i.e. during the early sperm aster stage and in the unfertilized egg. As the hyaline layer develops, eggs in normal sea water become progressively stiffer than naked eggs, the greatest absolute difference (13·8 units) occurring in mid-anaphase. In the later stages of cleavage the naked eggs give a higher stiffness value, but this is almost certainly due to the errors inherent in averaging on the rapidly falling curve. The naked eggs thus show the same general changes in stiffness as the eggs in ordinary sea water, except for the absence of the slow rise up to metaphase. One can assume therefore that this slow rise is due to the development of the hyaline layer, which acts as an additional elastic membrane on the surface of the cell.

Although we have only presented one set of results on the fertilized eggs of *P. miliaris*, we have made a large number of similar measurements on the eggs of this species and of *P. microtuberculatus* and *Paracentrotus lividus*. These have all shown changes in stiffness similar to those illustrated in Fig. 1.

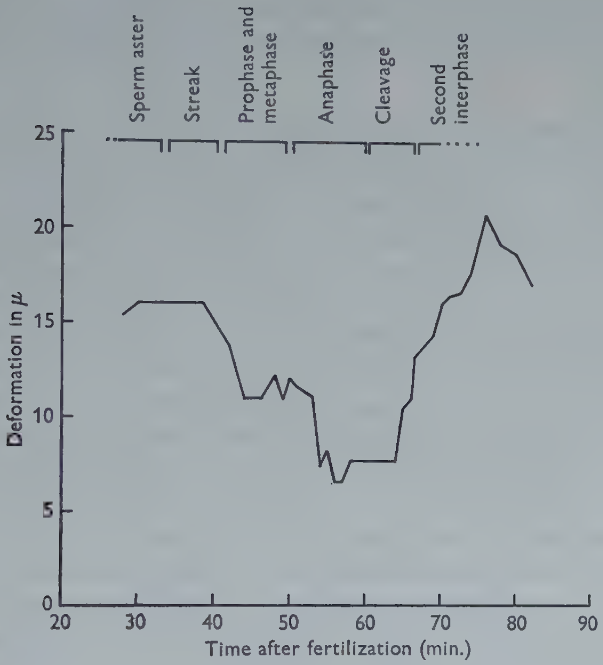


Fig. 2. Changes of deformation with constant pressure during first interphase and division. Egg of *Psammechinus miliaris*.

It might be expected, particularly at cleavage, that there would be variations in stiffness between different regions of the cell surface. We have looked repeatedly for such variations but have never found them at any stage, and there seems no doubt that the poles and the furrow regions of the dividing egg (at any rate during the early stages of cleavage) have similar mechanical properties at any given time. This is in agreement with the findings of Selman & Waddington (1955) on the very much larger eggs of amphibians. It is still possible, however, that the membrane in the depths of the furrow during late cleavage may have different mechanical properties, since this region cannot be reached with the elastimeter pipette.

An alternative method of investigating the changes in the membrane, is to keep an egg on the pipette with a constant pressure and follow the changes in deformation over a period of time. An example of such an experiment (a 'constant pressure run') is shown in Fig. 2, for a fertilized egg of *Psammechinus miliaris* in calcium-free sea water with a 41 μ pipette and a pressure of 6 dynes/cm.^{2}. The deformation here behaves in the opposite way to stiffness, and it can be seen that there is a marked drop in the deformation during anaphase and the early part of cleavage. The advantage of this method is that it is easy to get the exact time relations of the changes in the membrane. For instance, this particular example shows a characteristic feature of other constant pressure runs, namely that the reduction in deformation (corresponding to the increase in stiffness) starts during prophase and reaches its minimum value in the middle of anaphase, *before* the start of cleavage. These constant pressure runs, however, suffer from the serious disadvantage that they do not give quantitative values for stiffness, and for this reason we have not used them to any great extent.

STIFFNESS CHANGES AT FERTILIZATION

It is impossible to make direct measurements of stiffness during the fertilization of normal eggs because of the fertilization membrane. As soon as the eggs are fertilized the stiffness rises to a high value, and the effect of the cell membrane cannot be separated from that of the fertilization membrane which is much stiffer than the interphase egg. If the fertilization membranes are stripped off with bolting silk, measurements cannot be made until at least 3 min. after fertilization, since the membranes must be fully elevated before stripping, and this takes about 2 min. The only way round this difficulty is to use eggs where the elevation of the fertilization membrane has been inhibited by previous treatment with trypsin. Runnström, Monné & Broman (1943), who discovered this effect, believe that it is due to the action of trypsin in digesting away the vitelline membrane, which acts as the precursor of the fertilization membrane. Whether or not there is a morphologically separable vitelline membrane, it is certainly true that trypsin causes a large and irreversible drop in the membrane stiffness of unfertilized eggs (down to $\frac{1}{4}$–$\frac{1}{5}$ of normal eggs). This effect will be described in more detail in a subsequent paper on the effect of chemical agents on the unfertilized egg. After fertilization, trypsin-treated eggs develop and cleave normally.

Experiments were done with the eggs of *P. microtuberculatus* which had been treated, when unfertilized, with a solution of 0·1 % by weight of trypsin in sea water for 5 min. These eggs were fertilized on the elastimeter slide, and stiffness measurements made as soon as possible. All the eggs showed a sharp rise in stiffness, which reached a peak of 4–5 times the value of the unfertilized trypsin-treated eggs within the first 3 min. after fertilization. In the next 5 min. the stiffness fell back gradually to a value slightly below that for normal eggs at the sperm aster stage. Fig. 3 shows the results from four typical eggs, together with the corrected stiffness values for trypsin-treated unfertilized eggs, for normal unfertilized eggs, and for normal fertilized eggs at the sperm aster stage. The precise position of the height and timing of the peak value for stiffness is uncertain, since it is impossible to make instantaneous measurements at fertilization. The peak, therefore, may be higher and come nearer to the moment of fertilization than the graphs indicate. These results are incorporated in Fig. 1 as the dotted line in the first part of the curve.

It should be mentioned that constant pressure runs on trypsin-treated eggs give substantially similar results, with a drop in the deformation soon after fertilization.

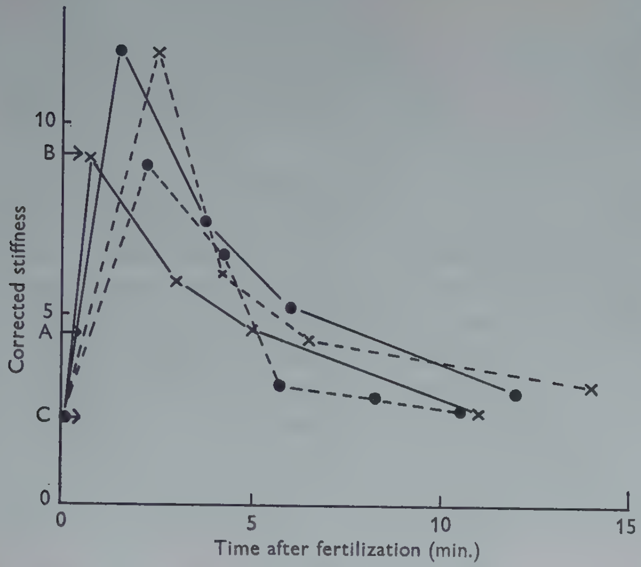


Fig. 3. Stiffness changes at fertilization. Four eggs of *Psammechinus microtuberculatus*. A, stiffness of normal unfertilized eggs; B, stiffness of unfertilized eggs treated with trypsin; C, stiffness of normal fertilized eggs at sperm aster stage. Corrected stiffness is in dynes/cm.^{2}/μ deformation for 100μ diameter egg and 50μ diameter pipette.

MEASUREMENTS ON SWOLLEN AND SHRUNKEN EGGS

For a correct interpretation of stiffness values, some knowledge of the internal pressure of the egg is needed. This problem is discussed in the second paper of the series, on the unfertilized egg (Mitchison & Swann, 1954*b*), and the reader should refer to this paper for a fuller description of the methods which can be used to give an indication of internal pressure. Only a summary of the methods will be given below.

It should be possible to determine whether or not an egg has an internal pressure (and, therefore, a resting tension in the membrane) by measuring the stiffness when the egg has been shrunk in a hypertonic solution. If there is pressure initially, the stiffness should fall, while if there is no pressure the stiffness should remain more or less constant. In either case, if the egg is swollen in a hypotonic solution the stiffness should rise.

Stiffness measurements on swollen or shrunken eggs must be made at a stage when the normal stiffness is known fairly accurately. It is only possible, therefore, to use two stages in the fertilized egg: the sperm aster stage, where the stiffness remains relatively constant for a long time; and the late anaphase or the 'wall-sided' stage of early cleavage, where the equatorial region of the egg is straight-sided and the exact stage of the egg is accurately known. Stiffness measurements were accordingly made on these two stages after the eggs (*P. microtuberculatus*) had been immersed for periods of 2–10 min. in the following media:

		Approx. molarity
'Full hypotonic'	50 ml. Ca-free sea water + 20 ml. dist. water	0·39
'Half hypotonic'	50 ml. Ca-free sea-water + 10 ml. dist. water	0·45
Normal Ca-free sea water		0·54
'Quarter hypertonic'	50 ml. Ca-free sea water + 5 ml. 2M-NaCl	0·67
'Half hypertonic'	50 ml. Ca-free sea water + 10 ml. 2M-NaCl	0·78
'Full hypertonic'	50 ml. Ca-free sea water + 20 ml. 2M-NaCl	0·96

The half and full hypertonic media were not used with the dividing eggs since they inhibited cleavage.

The results for late anaphase and early cleavage eggs are given in the first half of Table 2 and Fig. 4. It can be seen that there was a rise in stiffness, both in the hypotonic and the hypertonic media. Owing, however, to the rapid changes in stiffness that take place at this stage, there was a wide scatter in the measurements (see the last column of Table 2). A t test ($P=0{\cdot}05$) shows that, although the difference between the stiffness in full hypotonic and in normal sea water is significant, the other differences are not. We can conclude, therefore, that the cleavage eggs were behaving as though they had no internal pressure in ordinary sea-water; they showed a rise in stiffness when swollen, but no significant change in stiffness when shrunk.

The results for the eggs at the sperm aster stage are given in the second half of Table 2 and Fig. 5, and all the differences are significant ($P=0{\cdot}05$) except those between eggs in half hypertonic or half hypotonic and eggs in normal sea water. Although these results show a fall in stiffness in hypertonic media (as would be expected if there was an internal pressure in the normal egg at this stage), they also show a fall in hypotonic media. This experiment has been repeated twice, and in each case has given results similar to those in Table 2. The fall in stiffness in hypotonic media is an anomalous result if the egg is behaving like a simple elastic walled ball, since, whatever the normal pressure, the stiffness should increase when the egg is swollen. These results are discussed later (p. 744), and a possible explanation for them is given.

A direct method, of quite a different sort, was used to set an upper limit for the possible initial stretch of the membrane. The existence of an internal pressure implies, of course, that there is such an initial stretch. Eggs of *P. microtuberculatus*, at the sperm aster stage (15 min. after fertilization) and at late anaphase, were placed in hypertonic solutions of different strengths and were examined under the microscope after about 3 min. in order to find the smallest degree of hypertonicity which caused a visible wrinkling of the membrane. These solutions were found to be 50 ml. sea water + 5 ml. 2M-NaCl for the sperm aster eggs, and 50 ml. sea water + 15 ml. 2M-NaCl for the late anaphase eggs. Eggs from the same female were also photographed in these solutions and subsequently measured, together with controls in normal sea water. Figures are given in Table 3 for the mean diameters (mean of major and minor axes) of fifty eggs, for the standard deviation of the mean diameters and for the average ellipticity (major axis/minor axis).

Table 2. *Stiffness of swollen and shrunken eggs,* Psammechinus microtuberculatus

Stage	Medium	Molarity	No. of eggs	Average diameter (μ)	Corrected stiffness (dynes/cm.^{2}/μ deflexion for 100μ egg and 50μ pipette)	
					Mean	Standard deviation
Late anaphase and early cleavage	Full hypotonic	0·39	37	119	56·0	29·9
	Half hypotonic	0·45	19	115	36·5	22·5
	Normal	0·54	15	110	34·4	16·6
	Quarter hypertonic	0·67	18	105	45·9	19·8
Sperm Aster	Full hypotonic	0·39	16	119	2·04	0·76
	Half hypotonic	0·45	14	115	3·67	0·94
	Normal	0·54	5	110	5·04	0·89
	Half hypertonic	0·78	13	102	4·41	1·69
	Full hypertonic	0·96	15	96	2·41	0·36

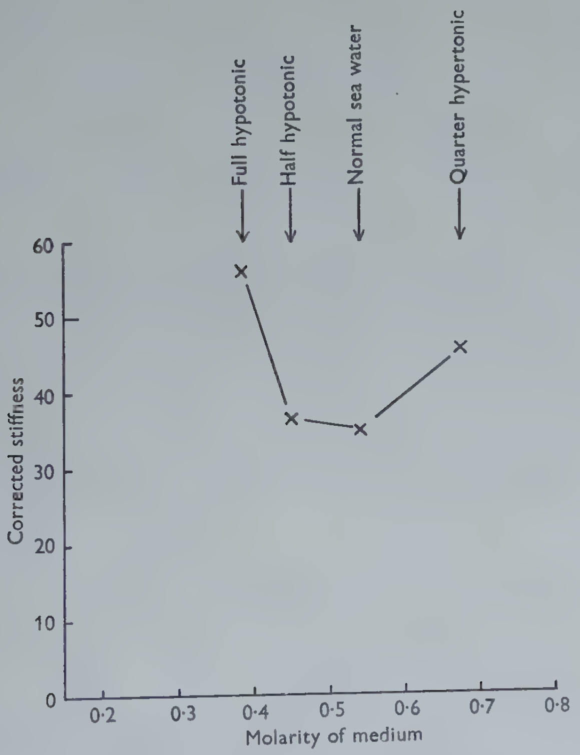


Fig. 4. Stiffness of swollen and shrunken eggs during later anaphase and wall-sided stages. Eggs of *Psammechinus microtuberculatus*. Corrected stiffness is in dynes/cm.^{2}/μ deformation for 100μ diameter egg and 50μ diameter pipette.

These results show that wrinkling first occurred when there had been a 3·5% linear shrinkage with the sperm aster eggs and a 9·0% shrinkage with the anaphase eggs. Since wrinkling cannot take place when there is an internal pressure, the membrane cannot normally be stretched by more than 3·7% (linear) from the resting state in the case of sperm aster eggs, and 9·7% in the case of anaphase eggs.

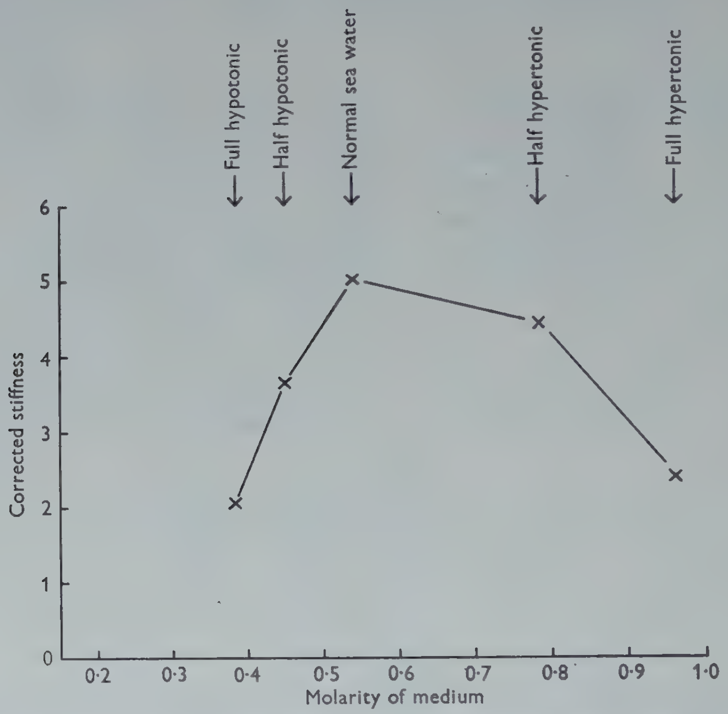


Fig. 5. Stiffness of swollen and shrunken eggs during the sperm aster stage. Eggs of *Psammechinus microtuberculatus*. Corrected stiffness is in dynes/cm.²/μ deformation for 100 μ diameter egg and 50 μ diameter pipette.

Table 3. *Wrinkling of fertilized eggs*, Psammechinus microtuberculatus

Stage	Medium	Average diameter (μ)	Standard deviation (μ)	Average ellipticity
Sperm aster	Normal sea water	113	5·5	1·025
Sperm aster	50 ml. sea water + 5 ml. 2M-NaCl	109	6·9	1·027
Late anaphase	50 ml. sea water + 15 ml. 2M-NaCl	103	11·3	1·038

By combining these figures with the stiffness values from Table 1 and with the data given in fig. 16 of the first paper, we can now set an upper limit for the possible internal pressure in normal eggs. During the early sperm aster stage, the maximum possible internal pressure is 19 dynes/cm.², which would reduce the value for Young's modulus from $0·58 \times 10^4$ to $0·48 \times 10^4$ dynes/cm.². During late anaphase, the maximum possible pressure is 500 dynes/cm.², and this would reduce the value for Young's modulus from $6 \cdot 81 \times 10^4$ to $4 \cdot 35 \times 10^4$ dynes/cm.^{2}. It must be emphasized that these pressure values are only upper limits and do not imply that there is necessarily any pressure at all. It may also be noted that there are at least two reasons for supposing that these experiments may overestimate the degree of shrinkage before wrinkling, and therefore the internal pressure. In the first place, the earliest signs of wrinkling are very difficult to detect under the microscope, and may occur with even smaller amounts of shrinkage than those found above. Secondly, it is probable that the initial effect of reducing the volume of a slightly elliptical body like an egg will be to increase the ellipticity rather than to produce wrinkling. Such an increase was in fact found in these experiments, as can be seen from Table 1.

DISCUSSION

I. *The experimental results*

The elastimeter measurements have shown that there are large changes in the stiffness of fertilized eggs during the division cycle. We have assumed that these represent changes in the elastic (Young's) modulus of the membrane, but there are three other factors which could also affect the measured stiffness. These are changes in the membrane thickness, changes in the internal pressure, and the existence of internal cytoplasmic rigidity, e.g. asters. Each of these factors will be discussed below.

It has been assumed, for the purpose of calculating Young's modulus, that the cortical thickness stays constant at about $1 \cdot 6\mu$. Changes in thickness would, of course, produce changes in the measured stiffness, but the thickness would have to increase about eightfold, i.e. from $1 \cdot 6$ to 12μ, to account for the twelvefold increase of stiffness between the sperm aster and the anaphase egg (see first paper, figs. 11 and 12). In fact there is no evidence at all of such a change, and Mitchison (1956) concludes that the membrane is roughly $1 \cdot 5\mu$ thick throughout the first interphase and division. The only evidence that conflicts with this is the observation by Chambers (1938*a*), that an oil drop in a cleaving *Lytechinus* egg is indented about 5μ ahead of the advancing furrow. This large figure for the membrane thickness may be due to different conditions in another species of sea urchin (Chambers finds that the experiment does not work with *Arbacia*), or it may simply be an overestimate caused by the difficulty of making accurate optical observations in the narrow furrow neck.

The presence of the asters (sperm aster and amphiaster) within the egg may also affect the elastimeter readings. From microdissection experiments asters are known to behave as discrete solid bodies, so that, unless care is taken, a deformation of the egg designed to measure the mechanical properties of the membrane may in fact be measuring the properties of the asters. This seemed to us a serious objection to Cole's (1932) method of measuring membrane properties by means of large compressions, and we designed the elastimeter to get over this difficulty as far as possible. The maximum deformation used with the elastimeter (a hemispherical bulge) only involves about a 5 % increase in the surface area of the membrane, and it is very unlikely that an aster could affect the readings unless it completely filled the whole cell and was attached to the membrane. Despite the arguments of Dan (1943), it seems that the asters are *not* attached to the surface since they can be moved about both by microdissection needles and by centrifuging (Harvey, 1935). It is also apparent in our own time-lapse films that the granules in the surface of a cleaving egg move independently of the asters. A further piece of evidence which suggests that the asters are not involved in the stiffness measurements is that the lowest stiffness values are reached during the sperm aster stage when there is an aster filling most of the cell. The stiffness only begins to rise markedly in metaphase when the sperm aster has disappeared and the amphiasters are so small that they could not affect the elastimeter readings. This is the exact reverse of what would be expected if the asters were responsible for the stiffness. Admittedly, the highest stiffness values are reached in late anaphase when the amphiasters are at their maximum size, but a similar rise in stiffness is shown at the equivalent time in eggs where the asters are suppressed by the action of colchicine (Swann & Mitchison, 1953). The peak stiffness of eggs in colchicine is not as high as in normal cleaving eggs, but it seems likely that colchicine weakens the membrane as well as destroying the asters. In conclusion, we believe that the asters are not affecting the stiffness measurements, though we cannot produce definite evidence at all stages. Ideally, we should like to make completely separate measurements on the membrane and the asters in a normal cleaving egg, but it would be difficult to do so.

The third factor which may affect the stiffness measurements, namely internal pressure, raises the most difficult problem of all. We have shown in the first and second papers that for any given stiffness value there is a series of solutions for Young's modulus and internal pressure varying from no internal pressure and a relatively high modulus to a high internal pressure and a relatively low modulus. The measurements on the wrinkling point set an upper limit to the possible internal pressure, and, in the case of the sperm aster stage, show both that the maximum possible pressure is very low (19 dynes/cm.^{2} or 1/50,000 of an atmosphere) and that there is only a small difference between the possible limits of the modulus ($0{\cdot}58-0{\cdot}48\times10^4$ dynes/cm.^{2}). In the case of the late anaphase eggs, however, although the limits of the modulus are still fairly close ($6{\cdot}81-4{\cdot}35\times10^4$), the maximum possible pressure is relatively large (500 dynes/cm.^{2} or 1/2000 of an atmosphere) because of the much higher absolute value of the modulus. This is not very informative if we wish to know the pressure changes at cleavage (which may be important in deciding between certain theories of cell division) since, as should be emphasized again, the method only sets an upper limit. The only other information about the internal pressure comes from the stiffness changes in hypo- and hypertonic media. With the anaphase and early cleavage eggs, the results seem to show that there is no internal pressure since there is no significant fall in the stiffness in hypertonic solution. Exactly comparable results were found for the unfertilized egg, as described in the second paper of the series. We have, however, to take account of the anomalous result with sperm aster eggs, which showed a fall in stiffness when swollen in hypotonic media. The most likely explanation of this result appears to be that the membrane itself is becoming hydrated and therefore weaker in hypotonic media, and that this weakness more than compensates for the increased stiffness caused by the stretching. Another possibility is that the weak membrane at this stage of development reaches a 'yield point', analogous to that described in the first paper, after only a small degree of stretch. In view of this effect, however, we do not feel justified in drawing any conclusions about the fall in stiffness of the shrunken sperm aster eggs in hypertonic media, which would otherwise indicate a positive pressure in the normal egg. Although these anomalous effects only occur when the membrane has the very low stiffness values characteristic of the sperm aster stage, they inevitably throw some doubt on the validity of the other stiffness measurements made in hypo- and hypertonic media on unfertilized and cleaving eggs.

Even if the maximum possible pressure were present within the eggs, it is clear that the Young's modulus of the membrane would still follow a curve very similar in shape to that given in Fig. 1. The peak value in late anaphase and early cleavage would be reduced by 36%, and the trough at the sperm aster stage would be reduced by 17%. We cannot say definitely what would happen to the values at other stages, but it seems very likely that they would be scaled down proportionately.

In conclusion, it is evident from the wrinkling experiments, that the maximum possible pressure at the sperm aster stage is very low, but it is difficult to be certain of the pressure in the cleaving egg. Wrinkling point experiments set a relatively high upper limit for pressure at cleavage, though stiffness measurements on shrunken eggs suggest that there is in fact no pressure at this stage. But the anomalous results of comparable experiments on the sperm aster stage throw some doubt on all the stiffness measurements on shrunken eggs.

The only means of settling this problem finally would be to make direct measurements of internal pressure, but the values are so low in absolute terms that this would be very difficult. Measurements have, in fact, been made of the internal pressure of other eggs by inserting into them a micropipette connected to a manometer, but they have mostly given very high and, we suspect, quite misleading values. We have found from our own experience that there are two sources of error in such measurements which are very difficult to overcome; the tendency of the cytoplasm to clog the pipette, and the inadequacy of the seal between the pipette and the cell surface.

II. *Earlier work on the mechanical properties of fertilized egg membranes*

One of the earliest attempts at measuring changes in the mechanical properties of the cell surface during the division cycle was that of Vlès (1926), who observed the degree of flattening of the sea-urchin egg under gravity, and calculated from this the surface tension using a 'sessile drop' formula. He found a decrease after fertilization and a sharp rise at about metaphase, followed by a fall during and after cleavage. His absolute values lie between 10 and 20 dynes/cm., and are 100 or more times greater than the values found by Cole (1932) and others. However, McCutcheon, Lucké & Hartline (1931), and we ourselves, have been unable to detect any flattening under gravity, while Harvey & Fankhauser (1933) have pointed out that the formula used by Vlès to calculate surface tensions is in any case wrong. It seems fairly certain, therefore, that Vlès's results are erroneous.

Harvey & Fankhauser (1933) applied the same method as Vlès to salamander eggs, though using another formula. They found a fall in tension at fertilization, with a slight rise before the first cleavage. But they mention that these results may be vitiated by changes in cytoplasmic gelation, and in the density of different regions of the egg. In any case, as Harvey & Danielli (1938) point out, no sessile drop formula is strictly applicable to a cell with an elastic membrane.

Cole & Michaelis (1932) applied Cole's (1932) gold-beam method to fertilized *Arbacia* eggs, but somewhat surprisingly found no changes during the mitotic cycle. There are, however, two reasons why these results cannot be regarded as conclusive. First, the experiments were done, as the authors point out, on unsatisfactory eggs at the end of the season. Secondly, this method of measurement involves large compressions of the eggs and is probably inaccurate when they contain asters.

The remaining attempts to measure the mechanical properties of the surface have involved centrifugation. Harvey (1933) measured the ease with which *Arbacia* eggs could be broken in two by centrifuging, and found that the strength of the surface declined within a few seconds of fertilization, continued to decline for some minutes, and then later rose again. This method, however, relies on first stratifying the cytoplasm, and is therefore sensitive to variations in cytoplasmic gelation as well as the strength of the surface. There is also an additional complication in *Arbacia* due to the increase at fertilization of the proportion of the dense pigment granules which are caught in the cortex. This should diminish the apparent ease of splitting. Somewhat similar experiments have been carried out by Heikens (1947) using *Limnaea* eggs. He found that 'tension at the surface', expressed as the ratio of breadth to length in centrifuged eggs, was low during polar body extension, and shortly before cleavage, but rose during and after cleavage. Here again the results are inevitably confused by the state of the cytoplasm and the ease with which it can be stratified.

A different technique was employed by Brown (1934), who measured the centrifugal force necessary to dislodge the pigment granules from the cortex of *Arbacia* eggs, and move them to one side of the cell. He expressed his results in terms of relative units of viscosity, though pointing out that this is not strictly appropriate. He found a small peak of viscosity in the early stages of mitosis, and a sharp rise just before cleavage.

Brown's results have been criticized by Wilson (1951), on the grounds that many of the pigment granules in *Arbacia* are not contained in the cortex, and that the method really measures a combination of cortical and cytoplasmic rigidity. Wilson himself has attempted to get over this difficulty by observing the cortical granules themselves in the eggs of *Chaetopterus*, rather than noting the appearance or otherwise of granules at the centrifugal pole of the egg. There were no very striking changes in cortical rigidity, though there was a slight fall after fertilization. Rigidity appeared to be constant before and after cleavage.

Marsland (1951) used the same criterion as Wilson for observing the changes in cortical rigidity in *Arbacia* eggs, though in some earlier papers he had used Brown's criterion. He found a tenfold increase in the centrifugal force required to displace the cortical granules in a cleaving egg as compared with an unfertilized egg. This is in agreement with the increase in rigidity at cleavage which we find with the elastimeter, but is in direct opposition to Wilson's results. The difference may be due to the material used, though it seems unlikely that there could be such a fundamental difference in the mechanical properties of two eggs which are fairly similar in size, appearance, and general behaviour.

It is not altogether clear what property is measured by the force necessary to shift cortical granules. It is likely to be related to rigidity, but, as Wilson points out, it is more akin to shear strength. It may perhaps bear some relation to the yield point we have noticed in our own experiments. In any case, it must be emphasized that the measurements will be sensitive to changes in size and density of the granules themselves, and it is perhaps wiser to suspend judgement on the results until we have a better idea of what mechanical property is being measured.

A short but interesting series of experiments was carried out by Danielli (1952), who compressed dividing sea-urchin eggs beneath a fragment of cover-slip, and found that shortly before cleavage they exerted sufficient force to raise it. He has interpreted this as a rise in membrane tension, but it should be pointed out that such a conclusion does not necessarily follow since the rise in Young's modulus which we have found, would produce the same result without any rise in tension or internal pressure. For example, a punctured tennis ball, compressed by a weight, will raise the weight if the Young's modulus of the rubber is increased by moderate heating. Since there is a hole in the ball, there can be no increase in the internal pressure. It should be mentioned at this point that the decrease in the deformation at cleavage during the constant pressure runs (p. 737) is analogous to Danielli's experiment.

It is evident that these experiments cannot be regarded as wholly satisfactory, since none of them make it possible to distinguish between tension and rigidity in the membrane. Nevertheless, there does seem to be a certain consensus of opinion that, as we have found, the strength of the cell surface declines during the first interphase, and increases at cleavage.

III. *Relevance to theories of cleavage*

A full discussion of the relevance of these results to the various theories of the mechanism of cleavage would take too much space for the present paper. Nevertheless, it will be apparent without going into details that the main result of this work, the rise in Young's modulus over most if not all of the cell surface at the time of cleavage, is not what would be expected on the basis of some of the theories.

For instance, theories that ascribe the active role in cleavage to the mitotic figure, and a merely passive one to the cell surface (e.g. Gray, 1924; Dan, 1943), would not be expected to lead to such striking changes in Young's modulus as we have found nor should they lead to changes beginning before the visible onset of cleavage. Still less would the surface be expected to become about ten times more rigid at the very moment when it has to be passively extended. Moreover, cleavage by this means should lead to a tension in the surface and the evidence, such as it is, points to there being no tension at this time. We believe also that our earlier work on cleavage without asters in the presence of colchicine (Swann & Mitchison, 1953) and on the timing of optical changes in the cell surface (Mitchison & Swann, 1952) provide strong evidence against this type of theory.

Somewhat similar arguments tell against the group of theories that suppose cleavage to be brought about by a contractile ring in the cell surface in the region of the furrow (e.g. Chambers, 1938*b*; Lewis, 1951; Marsland, 1951). There should, for instance, be evidence of different mechanical properties between the equatorial and polar regions of an egg that is cleaving, or about to cleave, but we have found no such evidence, nor have Selman & Waddington (1955) using our method on the very much larger amphibian egg. As before, it is highly improbable that the *whole* surface should get ten times stiffer at the very time when all but the equatorial ring has to be passively extended. The tension in the surface should also rise sharply, and while we have not been able to dispose of this possibility altogether, the evidence is certainly against it. Finally, there is our earlier work (Mitchison, 1952, 1953; Mitchison & Swann, 1952) on surface movements, optical changes and micro-dissection, which we believe also tells against any form of contractile ring theory.

The last group of theories depends on the expansion of the cell surface, either by growth (e.g. Schechtman, 1937; Selman & Waddington, 1955) or by molecular reorientation (Mitchison, 1952; Swann, 1952). Our present findings are not inconsistent with either of these two types of mechanism. Neither scheme should lead to an increase of tension in the surface. And while there is perhaps no obvious reason why growth should be associated with changes in rigidity, it might be argued that since growth is the result of an increased tendency to molecular aggregation, it should perhaps lead to an increased Young's modulus (compare, for example, the growth of the mitotic figure, or the growth of a crystal from the mother liquor). In the case of the 'expanding membrane' theory of cleavage, an increase in rigidity is very much to be expected, since the whole surface has to increase in area and force the furrow inwards.

Further discussion on the growth and expansion theories involves a detailed consideration of a number of points, and for this reason we propose to leave it to a later paper. It may be noted, however, that even on our own theory of expansion, there must be growth at some stage if the cell cortex is not to get progressively thinner. We are therefore inclined to consider the possibility of combining the two points of view, expansion by growth, and expansion by molecular reorientation, into a single theory. In any particular type of cell the contributions of the two mechanisms to cleavage would no doubt vary. In the case of the sea-urchin egg we believe that the evidence from the cell elastimeter, from surface movements and from optical changes all point to expansion by molecular reorientation over most of the egg surface being the more important process. In the very much larger amphibian egg, on the other hand, cleavage may be largely brought about by growth in the furrow.

SUMMARY

1. Measurements with the cell elastimeter on the stiffness of the cell membrane of fertilized sea-urchin eggs show the following general features. There is a sudden rise at fertilization, followed by a fall during the early sperm aster stage to the lowest value reached during development (a Young's modulus of about $0 \cdot 58 \times 10^4$ dynes/cm.^{2}). The stiffness rises slowly until metaphase, after which it rises rapidly to reach a maximum during late anaphase and early cleavage ($6 \cdot 81 \times 10^4$ dynes/cm.^{2}). During the later stages of cleavage the stiffness falls again and reaches a value in the second interphase which is about twice as high as in the first interphase. Measurements on naked eggs in calcium-free sea water indicate that the slow rise in metaphase is due to the development of the hyaline layer.

2. Measurements on swollen and shrunken eggs at cleavage indicate that there is no internal pressure in the eggs at this stage, but similar experiments with eggs at the sperm aster stage yield anomalous results. Observations on the wrinkling point in shrunken eggs show that the *maximum possible* internal pressure is 19 dynes/cm.^{2} for sperm aster eggs and 500 dynes/cm.^{2} for cleaving eggs.

3. The bearing of these results on various theories of the mechanism of cleavage is briefly discussed. The rise in Young's modulus of the whole cell surface at cleavage argues against theories depending on the action of the spindle and asters, and against theories proposing a contractile ring in the surface. The rise is, however, what might be expected on the basis of the expanding membrane theory.

Some of the work described in this paper was done while the authors were at the Department of Zoology, University of Cambridge, and it is a pleasure to acknowledge our thanks to Prof. Sir James Gray for his help and encouragement. We also wish to thank the Directors and Staff of the Marine Station, Millport, and the Stazione Zoologica, Naples, for their assistance in these experiments.

ADDENDUM

Since completing this work, we have read an interesting paper by Vlès (1933) on the mechanical deformation of sea-urchin eggs. Vlès used an apparatus which was similar in principle to our cell elastimeter, but he was concerned only with very great deformations when the egg was either largely or completely sucked up into the pipette. Using an approximate method, he arrived at a value of about 10^3 dynes/cm.^{2} for the Young's modulus of the cell surface of unfertilized eggs. This is about one-tenth of the value we have found, but, as Vlès himself pointed out, his method only gave an estimate of the order of magnitude of the modulus. He also made a series of measurements on the return of the deformed egg to its original shape, and some observations on fertilized eggs which had been deformed within their fertilization membranes.

REFERENCES

BROWN, D. E. S. (1934). The pressure coefficient of 'viscosity' in the eggs of *Arbacia punctulata. J. Cell. Comp. Physiol.* **5**, 335–46.

CHAMBERS, R. (1938*a*). Structural aspects of cell division. *Arch. Exp. Zellforsch.* **22**, 252–6.

CHAMBERS, R. (1938*b*). Structural and kinetic aspects of cell division. *J. Cell. Comp. Physiol.* **12**, 149–65.

COLE, K. S. (1932). Surface forces of the *Arbacia* egg. *J. Cell. Comp. Physiol.* **1**, 1–9.

COLE, K. S. & MICHAELIS, E. (1932). Surface forces of fertilised *Arbacia* eggs. *J. Cell. Comp. Physiol.* **2**, 121–6.

DAN, K. (1943). Behaviour of the cell surface during cleavage. VI. On the mechanism of cell division. *J. Fac. Sci. Tokyo Univ.* IV. **6**, 323–68.

DANIELLI, J. F. (1952). Division of the flattened egg. *Nature, Lond.*, **170**, 494.

GRAY, J. (1924). The mechanism of cell division. *Proc. Camb. Phil. Soc. Biol. Sci.* **1**, 164–88.

HARVEY, E. B. (1933). Effects of centrifugal force on fertilised eggs of *Arbacia punctulata* as observed with the centrifuge-microscope. *Biol. Bull., Woods Hole*, **65**, 389–96.

HARVEY, E. B. (1935). The mitotic figure and cleavage plane in the egg of *Parechinus microtuberculatus*, as influenced by centrifugal force. *Biol. Bull., Woods Hole*, **69**, 287–97.

HARVEY, E. N. & DANIELLI, J. F. (1938). Properties of the cell surface. *Biol. Rev.* **13**, 319–41.

HARVEY, E. N. & FANKHAUSER, G. (1933). The tension at the surface of the eggs of the salamander *Triturus (Diemyctylus) viridescens. J. Cell. Comp. Physiol.* **3**, 463–75.

HEIKENS, M. C. (1947). Viscosity changes during cleavage in the eggs of *Limnaea stagnalis. Proc. K. Akad. Wet. Amst.* **50**, 789.

LEWIS, W. H. (1951). Cell division with special reference to cells in tissue cultures. *Ann. N.Y. Acad. Sci.* **51**, 1287–94.

MCCUTCHEON, M., LUCKÉ, B. & HARTLINE, H. K. (1931). The osmotic properties of living cells (eggs of *Arbacia punctulata*). *J. Gen. Physiol.* **14**, 393–403.

MARSLAND, D. A. (1951). The action of hydrostatic pressure on cell division. *Ann. N.Y. Acad. Sci.* **51**, 1327–35.

MITCHISON, J. M. (1952). Cell membranes and cell division. *Symp. Soc. Exp. Biol.* **6**, 105–27.

MITCHISON, J. M. (1953). Microdissection studies on sea-urchin eggs at cleavage. *J. Exp. Biol.* **30**, 515–24.

MITCHISON, J. M. (1956). The thickness of the cortex of the sea urchin egg and the problem of the vitelline membrane. *Quart. J. Micr. Sci.* (In the press.)

MITCHISON, J. M. & SWANN, M. M. (1952). Optical changes in the membranes of the sea-urchin egg at fertilization, mitosis and cleavage. *J. Exp. Biol.* **29**, 357–62.

MITCHISON, J. M. & SWANN, M. M. (1954*a*). The mechanical properties of the cell surface. I. The cell elastimeter. *J. Exp. Biol.* **31**, 443–60.

MITCHISON, J. M. & SWANN, M. M. (1954*b*). The mechanical properties of the cell surface. II. The unfertilized sea-urchin egg. *J. Exp. Biol.* **31**, 461–72.

RUNNSTRÖM, J., MONNÉ, L. & BROMAN, L. (1943). On some properties of the surface layers in the sea-urchin egg and their changes upon activation. *Ark. Zool.* **35** A, no. 3, 1–32.

SCHECHTMAN, A. M. (1937). Localised cortical growth as the immediate cause of cell division. *Science*, **85**, 222.

SELMAN, G. & WADDINGTON, C. H. (1955). The mechanism of cell division in the cleavage of the newt's egg. *J. Exp. Biol.* **32**, 700–33.

SWANN, M. M. (1952). The nucleus in fertilization, mitosis and cell division. *Symp. Soc. Exp. Biol.* **6**, 89–104.

SWANN, M. M. & MITCHISON, J. M. (1953). Cleavage of sea-urchin eggs in colchicine. *J. Exp. Biol.* **30**, 506–14.

VLÈS, F. (1926). Les tensions de surface et les déformations de l'œuf d'oursin. *Arch. Phys. Biol.* **4**, 263.

VLÈS, F. (1933). Recherches sur une déformation mécanique des œufs d'oursin. *Arch. Zool. exp. gén.* **75**, 421–63.

WILSON, W. L. (1951). The rigidity of the cell cortex during cell division. *J. Cell. Comp. Physiol.* **38**, 409–15.

SEASONAL VARIATIONS IN THE GROWTH RATE, THYROID GLAND ACTIVITY AND FOOD RESERVES OF BROWN TROUT (*SALMO TRUTTA* LINN.)

By D. R. SWIFT

Freshwater Biological Association, The Ferry House, Far Sawrey, Ambleside

(*Received* 22 April 1955)

INTRODUCTION

Although it is an accepted fact that the rate of growth in salmonid fishes varies at different times of the year, the exact time and form of these fluctuations has never been precisely established. Allen (1940) and Cooper (1953) measured samples of fish from a large wild population, but under these conditions sampling errors made it difficult to assess accurately the differences in growth rate which occurred over small intervals of time, and extremely difficult to obtain information about the concurrent changes in the external environment. In the work of Pentelow (1939) and Wingfield (1940), who kept fish under laboratory conditions, the external environmental changes could be accurately assessed, but the numbers of fish used were small and the living conditions artificial.

The present work was undertaken to provide information for an investigation of the factors controlling the growth of fish. It was realized that the fundamental information which was required for this work was an accurate picture of the natural growth rate rhythm of the fish, and the time relationship between this and fluctuations in the external and internal environments. It seemed that the way to obtain this information was to work with as many fish as possible, kept under conditions as natural as they could be made without preventing accurate measurement of any fluctuations occurring in the environment.

These requirements were met by keeping a large population of fish in a stew-pond at the Freshwater Biological Association's hatchery, where the fish were subjected to all the naturally occurring fluctuations in temperature, chemical composition of the water, and day length, as were fish living in local streams. Other environmental factors, which according to previous workers, are liable to influence the growth rate, but which may vary locally, such as the kind and amount of food, the crowding and size relationships of individuals, and the varying state of sexual maturity throughout the population could, to a large extent, be controlled, and thus the possible varying effects of these factors on the growth rate of the fish eliminated. It was hoped by this means to obtain what could be regarded as a generalized growth rate curve for the trout living in this area under the conditions recorded. Trout were used for this work since (1) most previous work of this nature has been done on these fish, (2) they are readily obtainable of a known age and (3) they are easily kept in a hatchery. Fish which were 3 years old in the spring of the year of the work were used, thus enabling information to be obtained on the maturing of the gonads.

In order to obtain a guide to the seasonal changes occurring in the internal environment, a study of the fluctuations of the food reserves of these fish, obtained by investigating the protein, water, fat and glycogen content of the muscle, liver, gut wall and gonad was undertaken.

The role played by the thyroid gland in teleost metabolism has been the subject of many workers' attention. The control of the metabolic activity by the thyroid in mammals is well known, and many workers have tried to demonstrate a similar control in fish. This work has been reviewed by Hoar (1951) and Lyn & Wachowski (1951). All workers had negative results with the exception of Smith & Matthews (1948), who, using extracts of parrot fish thyroid, induced an increase in oxygen consumption in *Bathystoma*. The work of Lieber (1936) on *Misgurnis fossilis*, Hoar (1939) on the salmon and Buchmann (1940) on the herring showed that it was reasonable to expect that the thyroid of the trout would vary seasonally in its activity. Recently, Barrington & Matty (1954) have demonstrated seasonal variations in the thyroid gland of the minnow. The methods used by previous workers to estimate the activity of the thyroid gland have either depended on histological techniques (Hoar, 1939; Buchmann, 1940), or on measurement of the amount of iodine in the blood of the fish (Fontaine & Leloup, 1952). Of these methods that of Fontaine and his co-workers is probably the better as it is felt, in agreement with Carter (1933), that 'neither the size nor the histological appearance of a gland is necessarily correlated with the amount of secretion which it is pouring into the circulation', recently Fontaine (1953) has expressed a similar opinion. It was felt that it would be useful if some estimation of the seasonal changes in the activity of the thyroid gland of the trout could be obtained and it was decided to try, if possible, to estimate the rate of turnover of iodine in the gland, by injecting radioactive iodine, Matthews (1947), La Roche (1950), Olivereau (1952) and Berg & Gorbman (1953) having shown that the teleost thyroid will concentrate the isotope. Radioactive iodine has been used by many workers to measure the activity of the thyroid, the principal technique being to kill the animal at a known time after injection and excise the thyroid gland for estimation of the amount of isotope iodine in it. The gland in teleosts is a diffuse organ closely associated with other tissue, and it is therefore impossible to dissect out a whole gland from these animals. In clinical research on cases of thyroid malfunction the iodine concentrated by the gland has been measured *in vivo* by a counter tube held against the neck. It was decided to adapt this method for estimating the thyroid activity of the fish. Since this work was started Myant (1953) has used a similar method to investigate the thyroid activity of the rabbit.

METHODS

Section 1. *Growth rate*

A population of 250 3-year-old hatchery-reared brown trout were kept in two stewponds each of 12·5 kilolitres capacity and were fed to satiation on a mixture of minced liver and 'Dog-e-Tox' dog food. At monthly intervals a random sample of fifty fish was withdrawn from the ponds with a seine net, the fish were anaesthetized in a 2% solution of urethane, were measured to the nearest millimetre to the fork of the tail and returned to the ponds.

To overcome the possible error in these results caused by a steadily decreasing population, due to sampling for other investigations and what was afterwards considered to be an insufficient feeding level, the work was repeated in 1954, using a population of 100 3-year-old hatchery-reared fish kept in one pond. Each month all the fish were separately measured as before and also weighed to the nearest gram. For the purpose of calculating the mean length, the fish lengths were arranged in groups to the nearest centimetre and the specific growth rate calculated from the formula $\frac{\ln L_2 - \ln L_1}{T_2 - T_1} \times 100$, T being calculated in weeks. The specific growth rate in weight was calculated in a similar fashion.

Section 2. Tissue analysis

At monthly intervals a random sample of 9 fish was taken from the ponds and brought to the laboratory; each fish was separately and similarly dealt with. The fish was killed by a blow on the head, weighed and measured, the abdomen was opened and the liver, gonads and gut removed, the gut was emptied and all the organs weighed to the nearest 0·1 g. A sample of muscle was taken from a standard position in the shoulder of the fish. Each organ was separately treated, being frozen with liquid oxygen in a mortar and then ground to a fine powder with a pestle. Samples of the powdered tissue were taken for estimation of total water, protein, fat and glycogen.

Water content

About 500 mg. of tissue were heated at 100° C. in a hot-air oven to a constant weight. The difference between the initial and final weights giving the amount of water present.

Protein content

Protein was estimated by the micro-Kjeldahl method. The dried tissue from the total water estimation was dissolved in a known volume of sulphuric acid and aliquots of the solution were taken and heated in a digestion flask with a pinch of catalyst (Chibnall, Rees & Williams, 1943). The cooled digest was washed with distilled water into a Markham micro-Kjeldahl distillation apparatus, and 10 ml. of 40% sodium hydroxide solution were added. The ammonia distilled over was trapped in a 2% boric acid solution and was estimated by titration with 0·01 N hydrochloric acid, using as indicator a solution containing 0·02% methyl red and 0·1% brom cresol green. The amount of protein in the original samples was calculated from the nitrogen content using a factor of 6·25.

Fat content

About 500 mg. of tissue were placed in a 100 ml. flask with 50 ml. of 3:1 ethyl alcohol: ethyl ether mixture; the flask was refluxed for 3 hr. in a water-bath at 70° C. The tissue was then filtered off by a Whatman no. 1 filter-paper and the filtrate evaporated to dryness under reduced pressure in a water-bath at 50° C. The residue after evaporation was re-extracted with 15 ml. of petroleum ether b.p. 70–80° C. for 10 min. at 50° C., the extract being then washed through the original filter-paper with more petroleum ether into a 30 ml. beaker. Finally the petroleum ether was evaporated off in a water-bath at 50° C., and the residue, regarded as fat, was estimated by a weight difference, final traces of solvent being previously expelled in a vacuum desiccator containing calcium chloride and paraffin wax.

Glycogen content

Glycogen was seperated using the method of Good, Kramer & Somogyi (1933). Between 10 and 500 mg. of tissue were placed in a 15 ml. round-bottomed Pyrex centrifuge tube and 2 ml. of 30% potassium hydroxide added. The approximate amount of tissue taken varied with the type of tissue, so that the amounts of sugar to be estimated were kept roughly within the same limits; thus about 10 mg. of liver and about 500 mg. of muscle were used. The tubes were placed in boiling water until the tissue was dissolved, and after partial cooling the glycogen was precipitated by the addition of 2·2 ml. of 95% ethyl alcohol. The mixture, after being heated to boiling-point in a water-bath, was cooled and then centrifuged, the supernatant decanted off, and the tube inverted over filter-paper to drain. The remaining alcohol was expelled by placing the tube in a boiling-water bath. The glycogen was hydrolysed with 2 ml. of N sulphuric acid by heating in boiling water for 3 hr. The cooled solution was neutralized with N sodium carbonate and aliquots were taken for sugar estimation. The reagent described by Somogyi (1937) was used to determine the amount of sugar present, the excess iodine being titrated with 0·005 N sodium thiosulphate. The glycogen content was calculated from the glucose by using the factor 1·11.

Thyroid activity

Nine fish were taken at monthly intervals from the population at the hatchery and brought to the laboratory. Each fish in turn was anaesthetized in a 2% solution of urethane, marked by clipping a fin, weighed, and injected intraperitoneally via the anus, with a carrier-free solution of ^{131}I. The amount injected was such that each fish received approximately 0·1 μc./g. of fish. The fish were then returned to the hatchery.

At 6, 24, 48 and 72 hr. after injection the fish were brought to the laboratory, anaesthetized with urethane and placed over a lead shielded end-window type Geiger-Muller tube (G.E.C. type G.M.4). The fish was placed with the thyroid area over the end window (Fig. 1) and left in place for the duration of a thousand counts or 1 min., whichever was the shorter. After counting the fish were allowed to recover in running water and then returned to the hatchery. The results, recorded as counts per minute, corrected for the background count and the natural decay of the isotope, were plotted on a logarithmic scale against the time after injection of the fish. The regression coefficient, expressing the slope of the line fitted to these points, was taken as the index of thyroid activity.

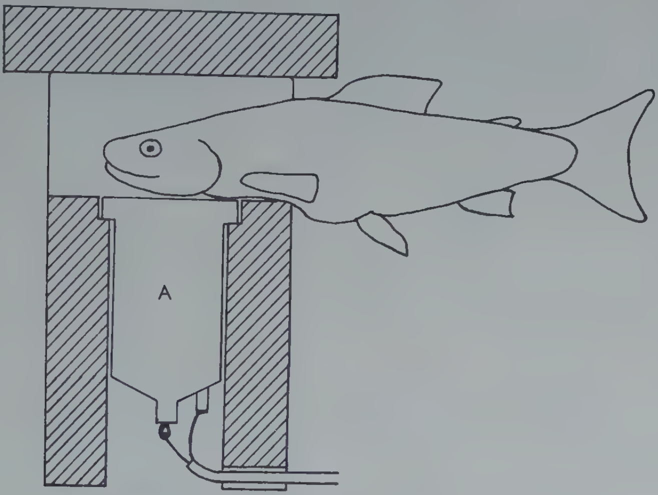


Fig. 1. Section through the lead castle showing the relative positions of the experimental fish and the Geiger–Muller tube (A).

The average monthly temperature of the hatchery water was calculated from a daily evening and morning measurement. Day length was calculated as a monthly average of the day lengths recorded by a Moll thermopile on a Cambridge recorder (threshold value 0·004 cal./cm.^{2}/min.).

RESULTS

The specific growth rate during 1954 (Fig. 2) follows the same general outline as that shown by the results for 1953 (Fig. 3). The 1954 results, however, are perhaps the more accurate ones as the 1953 curve is based on samples of fifty fish from a population which was steadily decreasing as monthly samples were taken for other work. That the fish were more intensively fed during 1954 than in 1953 is reflected in the greatly increased growth rate, but both curves are essentially the same in character. During the winter the fish grew very slowly, the specific growth rate in weight for this period (Fig. 4) reveals that the fish actually lost weight in January and February. In the spring the rate increased to reach a peak towards the end of April and then fell during May and June, increased to a new peak in August and decreased during autumn to the low winter level. A comparison of Figs. 2 and 4 shows that the specific growth rate in weight varies seasonally in a similar manner to that of length always, however, preceding it, thus the summer minimum period of growth in weight occurs during May.

The tissue analysis results are given in Table 1. The muscle and liver, apart from the liver glycogen content of the females which decreased in autumn, remained remarkably constant throughout the year. This is in marked contrast to the herring, the muscle and liver of which, according to Channon & El Saby (1932), vary seasonally in their fat contents and would seem to act as food reserve stores. The gut wall of the trout, the fat content of which varied considerably over the year, is the

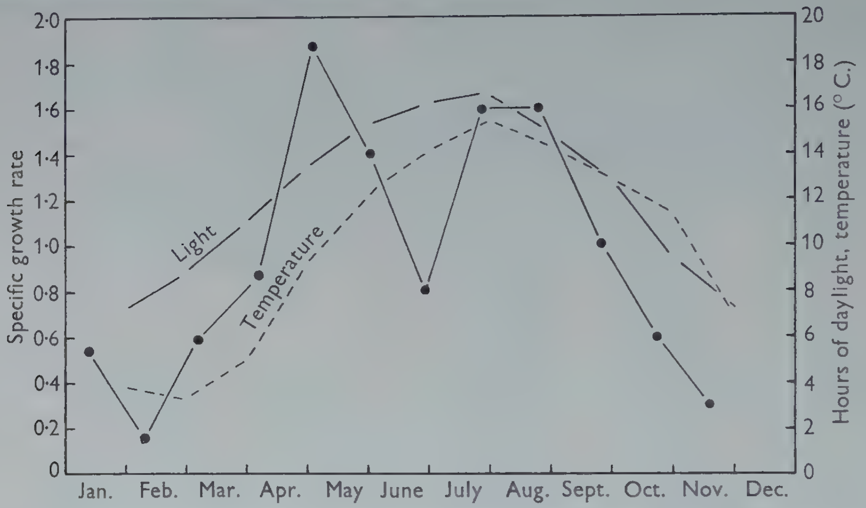


Fig. 2. The seasonal variations of specific growth rate in length during 1954. The growth rate is expressed as the percentage increase per week calculated from the formula $\frac{\ln L_2 - \ln L_1}{T_2 - T_1} \times 100$. The results were obtained by measuring the whole of the population.

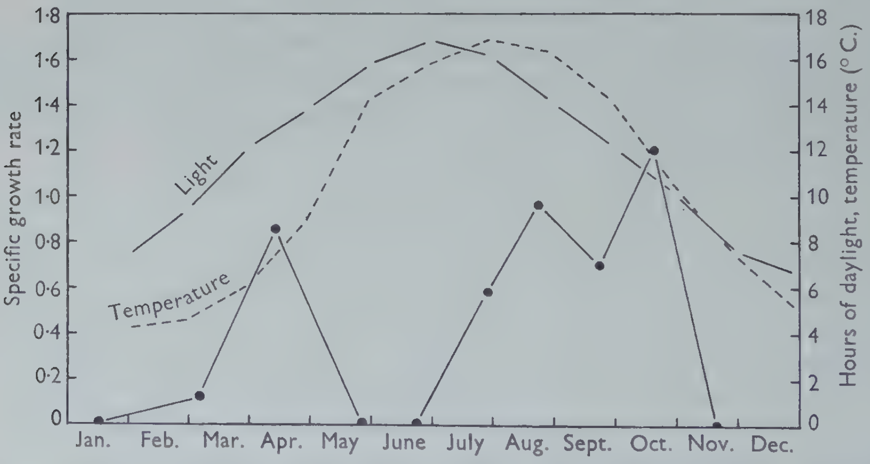


Fig. 3. The seasonal variations of specific growth rate in length during 1953. The growth rate is expressed as the percentage increase per week calculated from the formula $\frac{\ln L_2 - \ln L_1}{T_2 - T_1} \times 100$. The results were obtained by measuring random samples of 50 fish.

principal storage organ for fat which is the main food reserve of these fish. The immature fish in the autumn of 1951 had a fat content of about 12 % by weight (Table 1), during the winter this rose to about 20 %. This winter increase in reserves was probably due to the unnaturally abundant food in the hatchery. During the spring and early summer the fat reserves decreased to about 15 % in June, then increased very rapidly to a peak of 23 % in July. Throughout the late summer and early autumn they were heavily drawn upon so that by October they had decreased in the now ripe fish to a very low level of 5 %. Glycogen was detected in small amounts in the gut, muscle and gonad indicating that this compound is not stored there. The maturation of the gonads commenced during June in the females, and a little later in the males (Table 1). Ripening proceeded steadily until completion in September and October. The protein content rose 16 % in the ovary and 10 % in the testis, and the ovary fat level rose 3 %. In both the gonad and gut the water content varied inversely with the fat and protein.

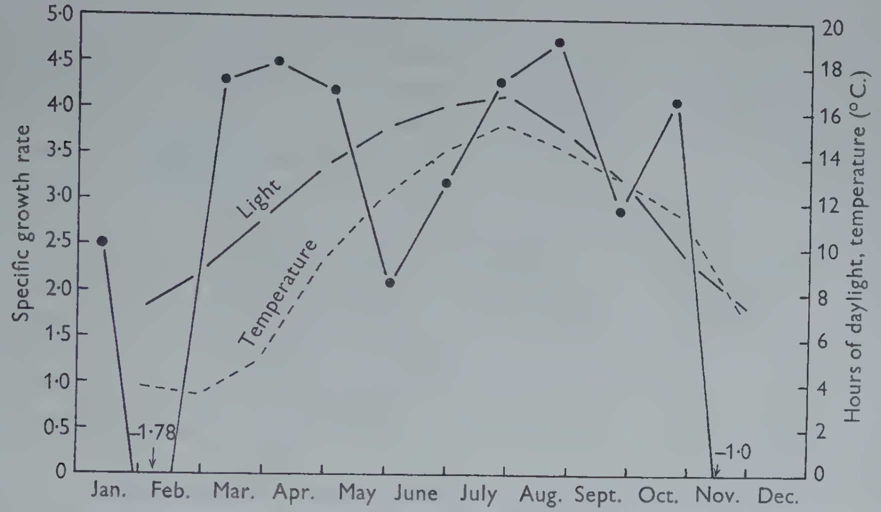


Fig. 4. The seasonal variations of specific growth rate in weight during 1954. The growth rate is expressed as the percentage increase per week calculated from the formula $\frac{\ln W_2 - \ln W_1}{T_2 - T_1} \times 100$. The results were obtained by measuring the whole of the population.

When isotope iodine is injected into a fish it enters the blood stream and then it is excreted by the kidneys and gut, or else it is trapped by the thyroid. On entering the thyroid the isotope iodine is probably very quickly distributed throughout the gland, so that some of the isotope may be immediately secreted in organic combination. However, it seems certain that more isotope iodine will, at this stage, be entering than leaving the gland and so a peak concentration of isotope in the gland is reached. After this peak has been reached the isotope content of the gland falls, showing that the rate of secretion in organic combination must now exceed the rate of uptake of isotope from the blood, some isotope presumably returning to the gland in inorganic form after being split from its organic combination. Thus the

Table 1. *Percentage composition by weight of the fish tissues*

	October	November	January	February	March	April	June	July	September	October	December
Muscle											
Water	77·8 (6)* 0·4†	78·8 (7) 0·4	77·8 (5) 0·4	77·8 (8) 0·4	80·0 (8) 1·2	77·7 (6) 0·5	79·1 (9) 0·6	77·2 (9) 0·4	78·4 (9) 0·3	79·2 (9) 0·4	79·1 (9) 0·4
Protein	18·4 (5) 0·7	18·6 (7) 0·4	17·7 (5) 1·1	18·7 (8) 0·2	17·8 (8) 0·9	19·3 (6) 0·2	18·8 (9) 0·7	19·7 (9) 0·5	19·4 (9) 0·3	18·0 (9) 0·7	17·6 (9) 0·4
Fat	2·5 (6) 0·3	2·3 (6) 0·3	4·5 (4) 1·4	2·8 (8) 0·2	2·7 (8) 0·3	2·7 (8) 0·3	2·3 (9) 0·2	2·6 (9) 0·2	2·2 (9) 0·1	2·3 (9) 0·2	2·9 (9) 0·4
Glycogen	— —	— —	0·4 (5) 0·08	0·2 (5) 0·06	0·6 (8) 0·09	0·2 (9) 0·03	0·1 (7) 0·002	0·2 (9) 0·04	0·2 (9) 0·04	0·2 (9) 0·04	0·5 (9) 0·08
Gut											
Water	70·7 (6) 2·5	71·1 (7) 2·1	66·4 (5) 1·4	64·4 (8) 2·2	68·1 (7) 2·4	68·2 (6) 3·0	68·6 (9) 2·1	61·8 (9) 2·6	72·3 (9) 0·9	75·8 (9) 1·9	77·9 (8) 0·9
Protein	16·2 (6) 2·0	13·8 (7) 0·5	12·0 (5) 0·7	11·8 (8) 0·4	13·6 (7) 0·05	13·7 (6) 1·0	14·3 (9) 1·1	13·2 (9) 0·6	15·0 (9) 0·5	14·6 (9) 0·6	14·2 (8) 1·1
Fat	11·3 (6) 0·9	11·0 (5) 0·8	19·9 (5) 2·4	20·5 (8) 1·9	16·1 (8) 2·6	14·4 (9) 2·3	14·6 (9) 2·0	21·4 (9) 2·7	10·7 (9) 0·8	5·2 (9) 0·9	6·1 (9) 0·6
Glycogen	—	—	0 (5)	0 (5)	0 (9)	0·04 (9)	0·02 (9)	0·03 (9)	0·02 (8)	0 (9)	0 (9)
Liver											
Water	75·5 (6) 0·7	74·7 (7) 0·7	77·2 (5) 2·5	75·9 (8) 0·5	76·1 (8) 0·4	76·5 (6) 0·5	79·1 (9) 0·6	75·4 (9) 0·4	77·2 (9) 0·6	76·8 (9) 0·7	76·8 (9) 0·6
Protein	15·8 (4) 1·2	15·7 (7) 0·5	14·4 (4) 0·3	15·4 (8) 0·4	17·3 (8) 1·1	17·7 (6) 1·2	16·7 (9) 1·4	15·7 (9) 0·7	14·4 (9) 0·9	13·5 (9) 0·7	14·9 (8) 0·3
Fat	4·8 (5) 0·8	3·9 (7) 0·3	4·3 (5) 0·9	5·1 (8) 0·2	5·0 (8) 0·1	4·9 (9) 0·2	4·6 (8) 0·3	5·2 (9) 0·6	4·8 (9) 0·2	3·9 (9) 0·1	5·1 (9) 0·4
Glycogen	— —	2·6 (7) 0·6	2·6 (5) 1·2	2·2 (5) 1·0	4·0 (8) 0·8	2·5 (9) 0·6	2·7 (9) 0·6	5·5 (9) 1·4	♀ 0·8 1·2 2·2 ♂ 5·5 (6) 0·6	♀ 1·0 1·1 — ♂ 7·9 (6) 1·5	3·5 (9) 0·5
Gonad											
Water	82·0 (5) 2·1	84·2 (7) 0·7	86·0 (3) 1·5	83·6 (5) 0·8	86·2 (8) 1·0	82·4 (5) 1·9	84·3 (7) 0·8	♀ 76·1 (6) 2·7 ♂ 81·7 84·4 86·8	♀ 61·9 61·9 63·6 ♂ 83·6 (6) 1·8	♀ 64·2 63·5 64·3 ♂ 78·3 (6) 1·5	♀ 65·0 65·5 ♂ 78·5 (6) 1·0
Protein	13·0 (5) 2·2	12·7 (6) 1·1	11·4 10·7 12·4	11·8 (5) 0·9	10·9 (8) 1·1	15·0 (5) 1·1	12·7 (7) 1·8	♀ 16·7 (6) 1·5 ♂ 8·2 12·3	♀ 25·4 26·7 28·0 ♂ 17·4 (6) 1·0	♀ 26·2 26·4 26·8 ♂ 20·5 (6) 1·5	22·9 (8) 0·8
Fat	4·7 (4) 1·5	—	2·5	2·0 3·6	2·4 (5) 0·4	3·8 (6) 1·4	4·0 (6) 0·7	♀ 5·1 (6) 1·3 ♂ 2·1 2·1 2·6	♀ 8·0 8·4 9·4 ♂ 3·1 (6) 0·7	♀ 4·9 7·6 7·7 ♂ 2·7 (4) 0·4	♀ 7·4 7·6 ♂ 3·0 (6) 0·7
Glycogen	—	—	0 0 0·15	0 0	0·08 (8)	0·04 (9)	0·01 (8)	0·06 (9)	0·03 (9)	0·06 (5)	0·04 (8)

* The figure in brackets indicates the number of samples taken; where this is less than three all the results are given.

† Standard error of the mean.

curve of uptake and loss of isotope by the gland is a composite one formed by the difference at any time between the secretion and uptake of the isotope. The rate of fall of this curve is used as a comparative index of the activity of the gland. The more active the gland, the more rapidly the peak isotope concentration is reached, and the steeper the slope of the curve for the loss of isotope, as the isotope is secreted in organic combination at a greater rate. The results of the investigation into the thyroid activity are shown in Fig. 5, the regression coefficient for the iodine loss/time curve for each fish examined is given, together with the monthly mean, from which it is apparent that the peak thyroid activity in these fish occurred in mid-summer.

DISCUSSION

The rate at which an animal grows is the direct result of the interplay of many internal factors, and perhaps the simplest form of control imaginable would be for the growth rate to be directly governed by the amount of metabolites surplus to the animal's maintenance requirements. These requirements vary seasonally in a poikilothermic animal such as a fish, increasing with the rising temperature of the water in summer and decreasing in winter. The observed growth rate for brown trout (Fig. 2) belies this hypothesis at three periods of the year. Growth commences in spring while the water is still cold, the rate falls in summer and again in autumn when the water is warm.

Brown (1946) found that brown trout growing under constant environmental conditions had two temperature optima for growth, one between 7 and 9° C. and the other between 16 and 19° C. She suggests that, as the observed relationship between temperature and maintenance requirements follow a sigmoid curve, the activity of the fish rose to a maximum between 10 and 12° C., and decreased at higher temperatures; this decrease being enough to compensate for the increased basal metabolism at high temperatures, leading, with increasing temperature, to a depression followed by an increase in the growth rate. The results obtained in this work (Fig. 2) show two temperature optima for growth. In 1954 the maximum growth rate occurred between 8 and 12° C. and between 15 and 16° C., showing a remarkable agreement with those of Brown in spite of the difference in experimental conditions. During June and early July the fish were most active, difficult to handle and showed a higher mortality as a result of handling than at any other time of the year. This observation supports Brown's hypothesis that the activity of the fish rises to a maximum between the two growth rate maxima, and her explanation of the causes of the depression she observed in the growth rate will also account for that shown in this work. This increased activity presumably leads to an increase in the amount of metabolites needed for maintenance, and thus reduces the quantity available for growth, the rate of which falls in consequence. As this activity cannot be solely a temperature effect the cause must be sought elsewhere. Two other factors which could be responsible are exerting their maximum influence at this time of the year; the thyroid gland, which has reached its peak activity, and day-length which is at its maximum.

The thyroid function in teleosts has been investigated by many workers (Root & Etkin, 1937; Etkin, Root & Mofshin, 1940; Hasler & Meyer, 1942; Smith & Everett, 1943; Matthews & Smith, 1947. They have sought in vain to influence the rate of oxygen consumption in fish by the use of known mammalian thyroid stimulants and depressants, and thus to show that the function of the gland in fish is the same as that in mammals, namely to control the basic metabolic rate. A suggested reason for these negative results is that a specificity has evolved, rendering the fish insensitive to these preparations. Supporting evidence of this explanation is provided by the work of Smith & Matthews (1948) who obtained an increase in the oxygen consumption of *Bathystoma* when injected with parrot fish thyroid extract. On the other hand, Matty (1954), working with thyroidectomized dogfish, noted no decrease in the oxygen consumption of the experimental animals. This would have been fairly conclusive evidence that the thyroid, at least in elasmobranchs, does not influence the basic metabolism, if Matty had not detected protein bound iodine in the plasma of an experimental animal 6 weeks after thryoidectomy. Rats, injected with thyroid gland extracts of teleosts (Smith & Brown, 1952), and of elasmobranchs (Matty, 1954), show a positive increase in oxygen consumption, indicating that the fish gland secretes a hormone which can exert a similar effect to that of the mammalian gland.

Another approach to the problem of thyroid gland function, which has been made here and by other workers, is to survey the seasonal variations in the activity of the gland. The results of the present work show that the peak activity occurs during midsummer, but D. C. W. Smith informs me that he has found, according to histological criteria, that the thyroid gland of 1-year-old trout reaches a maximum activity in spring. There would seem to be two possible explanations for the discrepancy between these results; either that the thyroid gland in immature and maturing fish reaches its maximum activity at different times of the year or that, as has been suggested, a histological examination does not reveal the true biochemical state of the gland. Further work must be done to determine biochemically precisely what activity states of the gland are represented by the histological criteria used by previous workers.

Lieber (1936) found two peaks of activity in the gland of *Misgurnis fossilis*, one in May and the other in August, these periods preceding and following spawning in these fish. Hoar (1939) working with salmon parr found evidence of maximum activity in May. Buchmann (1940) found a seasonal activity in the gland of the herring which coincided with spawning. Barrington & Matty (1954) showed the peak thyroid activity in the minnow to occur in spring just previous to spawning. All these results suggest that the thyroid is most active immediately prior to gonad maturation. This fact has led Barrington & Matty to suggest that the thyroid function in fish is concerned with gonad activity. This hypothesis is supported by the results of their experiment (1952), which showed that minnows suffered a retardation in their gonad maturation when immersed in an 0·1 % solution of thiourea. However, even if such a relationship was shown to exist it may not be the complete thyroid function as Hoar (1939), Buchmann (1940) and von Hagen (1936), working with the salmon, herring and eel respectively, have shown that a marked thyroid activity occurs at metamorphosis. Fontaine & Callemand (1942) have also reported two periods of marked thyroid activity in the eel which correspond to changes in the environment, and the results reported here show that peak thyroid activity in the brown trout correspond to a period of great physical activity in the fish. Thus all the fish observed with a highly active thyroid have exhibited a common factor of great physical activity, from this fact it is postulated that the thyroid hormone in fish exerts a tonic effect on the whole nervous system of the animal, rendering the animal more responsive to external environmental stimuli. These observations would also be in accord with a raising of the basic metabolic rate at times of great thyroid activity, but such an influence of the thyroid hormone remains to be demonstrated. The external environmental factor or factors responsible for the seasonal variation in the activity of the thyroid is unknown, the results shown here (Fig. 5) suggest that day-length may play a part in determining this activity.

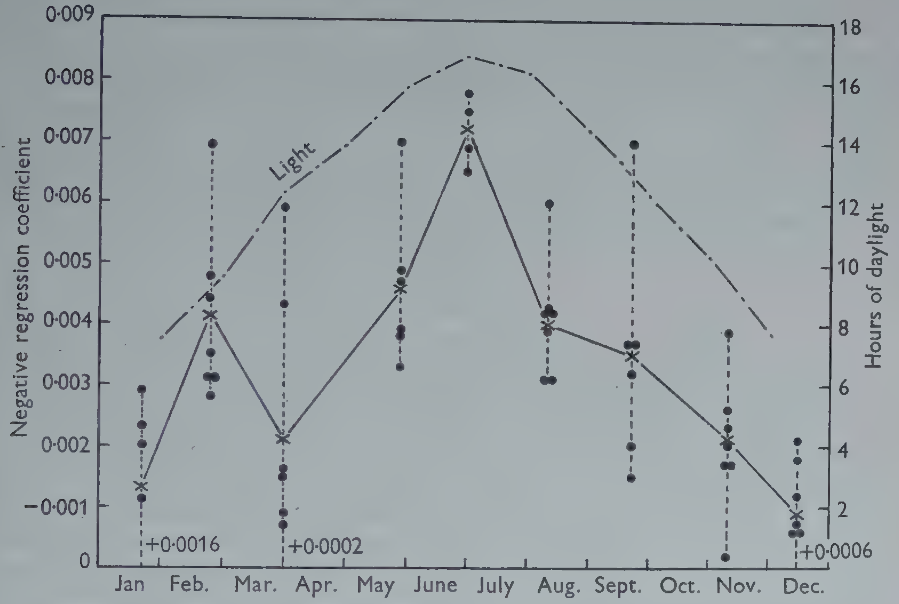


Fig. 5. Seasonal variations in the thyroid gland activity. The glandular activity is expressed by the regression coefficient for the iodine loss/time curve. The curve for the seasonal variation is drawn to the monthly means (×) of these regression coefficients.

At the beginning of this discussion it was pointed out that the observed growth rate of the fish differed from a proposed elementary relationship between temperature and growth at three periods, in spring, in summer and in autumn. The summer anomaly is caused, in all probability, by the increase in activity of the fish. The autumn anomaly is without doubt caused by the ripening of the gonads, which occurs during July, August and September (Table 1). During late summer when the rate of supply of metabolites from the gut, if dependent on water temperature, must be virtually at its maximum, the fish is able to maintain a high growth rate, ripen its gonads and lay down food reserves (Fig. 2, Table 1). By August it seems that this supply of metabolites is insufficient to maintain both growth and gonad maturation. This is inferred from the fact that by October the fat stores around the gut have dropped from the July figure of 22 to 5 % of the gut wall. During this period the females also draw on their liver glycogen stores. By the end of August the water temperature, and thus presumably the rate of supply of metabolites from the gut, begins to fall. The gonads are still ripening and their increased demand for metabolites from the decreasing supply available, is reflected by a fall in the growth rate. This interpretation is supported by a comparison of the growth rate curves for 1953 and 1954 (Figs. 2 and 3). In 1953 a very low percentage of the population ripened, and the fish maintained their growth rate until October, whereas in 1954 the growth rate of the fish, nearly all of which were ripe, decreased from the end of August.

That growth hormone occurs in fish (Pickford, 1954), suggests a possible cause of the commencement of growth in early spring, when this occurs the water temperature is still very low but day-length is increasing, suggesting that here again it is day-length which is the important external environmental factor.

SUMMARY

1. Seasonal variations in the growth rate, food reserves and activity of the thyroid gland of hatchery-reared brown trout have been investigated.

2. Two peaks of maximum growth rate were found, in spring and autumn. A marked depression of rate occurred during midsummer and winter.

3. Fat which was laid down along the mesenteries and pyloric caecae was found to be the main food reserve. Glycogen was found in small quantities in the liver and muscle. The composition of the muscle and liver was constant except for an autumnal fall in the female liver glycogen level. The fat reserves reached a peak of 23 % by weight of the gut wall during July then fell to 5 % in autumn.

4. Maturation of the gonads commenced in females in June and in males in July and was completed during October. The protein content of the ovary increased by 16 %, and of the testis by 10 %. The fat content of the ovary increased by 3 %.

5. A new method is described for the determination of thyroid activity in fish using radioactive iodine. Peak thyroid activity was found to occur in midsummer.

I wish to thank Prof. F. G. Young for allowing me to do the preliminary parts of this work in his laboratory, also the many people who helped me there, in particular Dr J. C. Boursnell for his advice on the handling of radioactive isotopes. I am grateful for the assistance given to me by various members of the Freshwater Biological Association laboratory staff, in particular that of Mr D. F. Parker. Miss C. Kipling gave me invaluable advice on statistics. I wish to thank Prof. R. J. Pumphrey and Dr C. L. Smith for much stimulating criticism and advice. Finally, I am indebted to Mr H. C. Gilson who has advised and encouraged me throughout this work.

REFERENCES

Allen, K. R. (1940). Studies on the biology of the early stages of the salmon. *Salmo salar.* 1. Growth in the river Eden. *J. anim. Ecol.* **9**, 1.

Barrington, E. J. W. & Matty, A. J. (1952). Influence of thiourea on reproduction in the Minnow. *Nature, Lond.*, **170**, 105.

Barrington, E. J. W. & Matty, A. J. (1954). Seasonal variation in the thyroid gland of the minnow *Phoxinus phoxinus* L. with some observations on the effect of temperature. *Proc. Zool. Soc. Lond.*, **124**, 89.

Berg, O & Gorbman, A. (1953). Utilization of iodine by the thyroid of the platyfish *Xiphophorus maculatus. Proc. Soc. Exp. Biol., N.Y.*, **83**, 751.

Brown, M. E. (1946). The growth of brown trout. (*S. trutta* L.). 3. The effect of temperature on the growth of two-year-old trout. *J. Exp. Biol.* **22**, 145.

Buchmann, H. (1940). Hypophyse und Thryoidea im Individualzyklus des Herings. *Zool. Jb.* **66**, 191.

Carter, G. S. (1933). On the control of the level of activity of the animal body. 1. The endocrine control of seasonal variations of activity in the frog. *J. Exp. Biol.* **10**, 256.

Channon, H. J. & Saby, M. K. El (1932). Fat metabolism of the herring. 1. A preliminary survey. *Biochem. J.* **37**, 354.

Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). The total nitrogen content of egg albumin and other proteins. *Biochem. J.* **37**, 354.

Cooper, E. L. (1953). Periodicity of growth and change of condition of brook trout (*Salvelinus fontinalis*) in 3 Michigan trout streams. *Copeia*, no. 2, 107.

Etkin, W., Root, R. W. & Mofshin, B. P. (1940). The effect of thyroid feeding on oxygen consumption of goldfish. *Physiol. Zoöl.* **13**, 415.

Fontaine, M. & Callemand, O. (1942). L'activite thyroidienne de L'Anguille au cours de son developpement. *Arch. Zool. exp. gén.* **82**, 129.

Fontaine, M. & Leloup, J. (1952). Iodemie et thyroxinemie elevees des poissons Teleosteens migrateurs amphibiotiques. *C.R.* 11° *Congres International Chim. Biol. Paris* 1953.

Fontaine, M., Leloup, J. & Olivereau, M. (1953). Da la necessite de l'emploi des techniques convergentes pour l'etude de la fonction thyroidienne chez les Poissons. *Congr. int. Physiol. Montreal Sept.* 1953.

Good, Kramer, & Somogyi (1933). The determination of glycogen. *J. Biol. Chem.* **100**, 485.

Hagen, F. von (1936). Die wichtigsten Endokrinen des Flusaals. Thyroidea, Thymus and Hypophyse im Lebenzyklus des Flusaals. (*Anguilla vulgaris*) nebst einegen Untersuchungen uber das chromophile und chromophobe Kolloid der Thyroidea. *Zool. Jb.* **61**, 467.

Hasler, A. D. & Meyer, R. K. (1942). Respiratory responses of normal and castrated goldfish to teleost and mammalian hormones. *J. Exp. Zool.* **91**, 391.

Hoar, W. S. (1939). The thyroid gland of the Atlantic salmon. *J. Morph.* **65**, 257.

Hoar, W. S. (1951). Some aspects of the physiology of fish. *Ont. Fish. Res. Lab.* no. lxxi.

La Roche, G. (1950). Fixation du radioiode par la glande thyroide du Saumon de l'atlantique. *Ann. ACFAS.* **16**, 134.

Lieber, A. (1936). Der Jahrezyklus der Schildrüse von *Misgurnis fossilis* L. und seine experimentelle Beeinflussbarkeit. *Z. wiss. Zool.* **148**, 364.

Lyn, W. G. & Wachowski, H. E. (1951). The thyroid gland and its function in cold blooded vertebrates. *Quart. Rev. Biol.* **26**, 123.

Matthews, S. A. (1947). Concentration of ^{131}I by the thyroid gland of the Bermuda Parrot Fish. *Anat. Rec.* **99**, 592.

Matthews, S. A., & Smith, D. C. (1947). The effect of thiourea on the oxygen consumption of Fundulus. *Physiol. Zoöl.* **20**, 16.

Matty, A. J. (1954). Thyroidectomy of the dogfish *Scyllium canicula* L. and the effect of dogfish thyroid upon the oxygen consumption of rats. *J. Mar. Biol. Ass. U.K.* **33**, 689.

Myant, N. B. (1953). The uptake of radio-iodine by the rabbit's thyroid measured in vivo. *J. Physiol.* **120**, 278.

Olivereau, M. (1952). Action de la thiouree et du thiouracil sur le comportement de l'iode radioactif dans la glande thyroide de deux teleosts marins. *C.R. Soc. Biol., Paris*, **146**, 248.

Pentelow, F. T. K. (1939). The relation between growth and food consumption in the brown trout. *S. trutta* L. *J. Exp. Biol.* **16**, 445.

Pickford, G. E. (1954). The response of hypophysectomized male killifish to purified fish growth hormone as compared with the response to purified beef growth hormone. *Endocrinology*, **55**, 274.

ROOT, R. W. & ETKIN, W. (1937). Effect of thyroxin on oxygen consumption of the toadfish. *Proc. Soc. Exp. Biol., N.Y.*, **37**, 174.

SMITH, D. & BROWN, F. (1952). The effect of parrot fish thyroid extracts on the respiratory metabolism of the white rat. *Biol. Bull., Woods Hole*, **102**, 278.

SMITH, D. C. & EVERETT, G. M. (1943). The effect of thyroid hormone on growth rate, time of sexual differentiation and oxygen consumption in the fish *Lebistes reticulatus*. *J. Exp. Biol.* **94**, 229.

SMITH, D. C. & MATTHEWS, S. A. (1948). Parrot fish thyroid extract and its effect upon oxygen consumption in the fish *Bathystoma*. *Amer. J. Physiol.* **153**, 215.

SOMOGYI, M. (1937). A reagent for the copper iodometric determination of very small amounts of sugar. *J. Biol. Chem.* **117**, 771.

WINGFIELD, C. A. (1940). The effect of certain environmental factors on the growth of brown trout. *Salmo trutta* L. *J. Exp. Biol.* **17**, 435.

THE WATER RELATIONS OF EARTHWORMS

I. THE ACTIVITY OF THE NEPHRIDIOSTOME CILIA OF *LUMBRICUS TERRESTRIS* L. AND *ALLOLOBOPHORA CHLOROTICA* SAVIGNY, IN RELATION TO THE CONCENTRATION OF THE BATHING MEDIUM

By BETTY I. ROOTS
*Department of Zoology, University College, London**

(*Received* 24 *March* 1955)

I. INTRODUCTION

The distribution of earthworms is incompletely known, particularly in relation to soil type, but it is now clear that the different species of earthworms show different soil preferences. This leads one to inquire whether there are physiological differences correlated with the distributional differences between species. This problem, with special reference to water content, forms the subject of this paper, particular attention being paid to the action of hypo- and hypertonic media on the cilia of the nephridiostome.

Both *Lumbricus terrestris* and *Allolobophora chlorotica* are relatively easy to obtain and were chosen for these experiments because of their contrasting ecological distribution. *L. terrestris* is purely terrestrial, but *A. chlorotica* occurs in soil but also submerged in streams and even in Lake Windermere up to 20 m. from the shore (Černosvitov, 1945).

II. MATERIAL AND METHODS

Material

A regular supply of *L. terrestris* was obtained from Newdigate, Surrey. Garden specimens of *A. chlorotica* were collected as required from south-east London. Both species were kept in pots of moist soil at temperatures ranging between 8 and 18° C., and were used within a week of collection. Lake Windermere specimens of *A. chlorotica* were kept submerged in a large tank at temperatures ranging between 8 and 15° C., and were used within a month of collection.

Preparation of nephridia

Living nephridia were dissected out and mounted on slides in the way shown in Fig. 1. Preparations mounted in this way were inverted over a pot of saline and observed through a microscope with a $\frac{1}{3}$ in. objective. By means of a vulcanite chamber with inlet and outlet tubes it was possible to observe the cilia while the bathing fluid was changed.

---

* Present address: Department of Biology, Royal Free Hospital School of Medicine, London, where some of the later experiments were made.

Preparation of the bathing medium

Adolph (1927) found that the coelomic fluid of *L. terrestris* had a Δ of 0·31° C. This was confirmed by Ramsay (1949*a*), who also estimated the chloride content and found that only half the osmotic pressure could be accounted for as chloride and suggested (1949*b*) that the other half is probably due to organic substances. Bahl (1945, 1946) analysed fully the coelomic fluid of *Pheretima posthuma* (Megascolecidae), but as the chloride content is half that found for *Lumbricus* it is clear that the composition of the fluid is different in the two species. Thus there is no standard earthworm Ringer solution. Therefore many solutions, based on the above information, were tested.

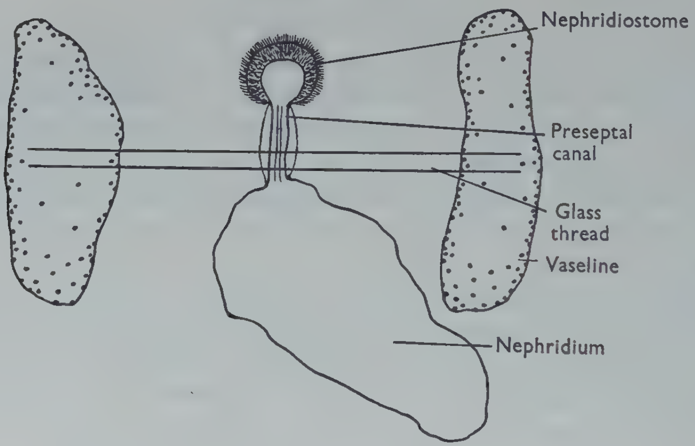


Fig. 1. Diagram to show method of mounting nephridia.

The two which gave the best results were frog Ringer* diluted with half its volume of M/400-$NaHCO_3$, and the same diluted Ringer mixed with an equal volume of 30 g./l. dextrose. Both fluids had a pH of 7·8–8·0 and a calculated Δ of 0·30° C. In one case the osmotic pressure was entirely due to salts; in the other, half to salts and half to dextrose. Full ciliary activity was maintained for about 22 hr. in the solution with dextrose (henceforth referred to as earthworm saline, or E.S.), whereas in the diluted frog Ringer alone ($\frac{2}{3}$ F.R.) full activity was maintained for only 16–20 hr.

As survival was curtailed at temperatures above 18° C. the experiments were made at temperatures ranging from 8 to 18° C., the majority being at 15–17° C., and the variation during any one experiment never exceeded 1° C.

---

* The frog Ringer had the following composition: NaCl 0·107 M, KCl 0·00165 M, $CaCl_2$ 0·00113 M, $NaHCO_3$ 0·0025 M.

III. RESULTS

(1) *Ciliary activity in hypotonic fluids*

After about an hour in the isotonic medium the nephridia were transferred to a hypotonic medium, dilutions being made with M/400-$NaHCO_3$. The activity of the cilia was noted periodically and compared with that of control preparations kept in undiluted media. As with other ciliated tissues the transfer of a nephridium to a new salinity often produces responses which gradually disappear as the tissue accommodates itself to the new medium. We must therefore distinguish between the temporary effects of the change and the long-term effects of a new tonicity.

(i) *Long-term effects of hypotonicity*

The duration of activity in the various hypotonic solutions is shown in Table 1. In any one column, the solutions are seen to fall into three groups:

(*a*) Those in which the dilution is slight; in these the cilia remain active for as long as in isotonic media, although they show a slight falling off in vigour in the last few hours.

(*b*) Those in which the dilution is intermediate; in these some of the individual preparations behave as in group (*a*) and the others as in group (*c*).

(*c*) Those in which the dilution is extreme; in these the preparations are rapidly arrested, the duration of activity varying inversely with dilution.

When the different columns are compared, the following conclusions clearly emerge:

(*a*) The resistance of the preparation to dilution depends on the basic saline, $\frac{2}{3}$ F.R., giving greater resistance than E.S. There is an approximate correspondence between the results for a given dilution of $\frac{2}{3}$ F.R. and those for double that concentration of E.S. As the salt concentration in E.S. is half that in $\frac{2}{3}$ F.R., these results suggest that salt concentration is more important than osmotic pressure in determining the long-term effects.

(*b*) In either medium, the nephridia of *A. chlorotica* are more resistant to dilution than those of *L. terrestris*.

(*c*) The nephridia of garden and Windermere specimens of *A. chlorotica* were compared in E.S. only, but no significant difference was observed in their long-term salinity relations.

(*d*) The cilia of *L. terrestris* and *A. chlorotica* are able to withstand a dilution of the medium much greater than they are likely to experience under natural conditions. Ramsay (1949*a*) has shown that *L. terrestris* regulates the osmotic concentration of its coelomic fluid, and variations are therefore unlikely to be very large.

(ii) *Temporary effects of change*

In both species the temporary effects were excitatory—acceleration of the beat or increased amplitude, or both together.

Generally there was a time lag of about 1 min. before these effects appeared, but in some cases they occurred within 4 sec. of transference. Four seconds was the time required to commence observations after transferring preparations.

The duration of the effects was variable and apparently bore no relation to the degree of dilution in either *L. terrestris* or *A. chlorotica* (Table 2). The results hardly allow a comparison between E.S. and ⅔ F.R. The only generalization that can be made is that in the higher concentrations there were no temporary effects and that in the lower concentrations they invariably occurred. In intermediate concentrations of ⅔ F.R. temporary effects occurred in some only of the preparations.

The results are essentially the same for the two species, and the results for garden and Lake Windermere specimens of *A. chlorotica* agree well.

The nephridiostome cilia of these earthworm species differ from the cilia of molluscan gills and the nephridial cilia of the polychaete *Arenicola marina*, in that there is no inhibition of the cilia after a sudden drop in osmotic pressure, the temporary effect being acceleration and increased amplitude of the beat. Wells, Ledingham & Gregory (1940) found that after a change from 100 to 20% or less a phase of excitation preceded the inhibition of *Mytilus* short abfrontal cilia. Pilgrim (1953) found that the frontal and food-groove cilia behaved in a similar way when subjected to a change from 100 to 50% or lower. Pilgrim also found that with smaller reductions in concentration the frontal cilia sometimes showed hyperactivity without inhibition.

(iii) *Return to isotonic media*

The effect of return to an undiluted medium was investigated in *L. terrestris* for 25 and 30% ⅔ F.R. and for 40% E.S. After 15 min. in the hypotonic medium preparations were transferred back to 100%.

In all cases about 15 sec. after transfer the activity of the cilia was reduced for a time, after which the activity returned to the normal level. Inhibition lasted for 3–8 min. when the change was from 25% ⅔ F.R.; for 2–12 min. when the change was from 30% ⅔ F.R.; and for 55 sec. to 8 min. when the change was from 40% E.S.

(2) *Ciliary activity in hypertonic fluids*

The experimental procedure was the same in these experiments as in those with hypotonic fluids but only E.S. was used. As before it is necessary to distinguish between the temporary and the long-term effects of a change.

(i) *Long-term effects of hypertonicity*

The duration of ciliary activity at various degrees of hypertonicity is shown in Table 3. As in the case of hypotonic fluids, one can distinguish between a slight change, which does not significantly shorten the duration of ciliary activity, an extreme change which rapidly suppresses movement altogether, and an intermediate change which sorts out the individual preparations into two groups. Even in those

Table 1. *Long-term effects of hypotonic media*

The first figure in each case is the time until activity ceased altogether. The second (in brackets) is the time until activity was noticeably less than that of a preparation in the undiluted medium. The third figure gives the number of preparations used, and the fourth the number of worms from which these were made.

Percentage of isotonic solution	⅔ frog Ringer						Earthworm saline								
	L. terrestris			*A. chlorotica* (garden)			*L. terrestris*			*A. chlorotica* (garden)			*A. chlorotica* (lake)		
	Duration	Preparations	Worms	Duration	Preparations	Worms	Duration	Preparations	Worms	Duration	Preparations	Worms	Duration	Preparations	Worms
80	> 20 hr. (16–19 hr.)	15	3	—	—	—	> 22 hr. (19–20 hr.)	15	3	—	—	—	—	—	—
60	—	—	—	—	—	—	> 22 hr. (19 hr.) 90 min. (20–40 min.)	18 17	5 5	—	—	—	—	—	—
50	> 20 hr. (16–19 hr.)	15	3	> 20 hr. (16–19 hr.)	15	3	1–2 hr. (15–50 min.) > 22 hr. (19 hr.)	26 14	6 4	> 22 hr. (19–20 hr.)	15	3	> 22 hr. (19–20 hr.)	23	4
40	> 20 hr. (16–19 hr.)	20	4	> 20 hr. (16–19 hr.)	15	3	1–2 hr. (15–30 min.) > 22 hr. (19 hr.)	28 7	5 3	> 22 hr. (19–20 hr.) > 22 hr. (5 hr.)	12 3	3 2	> 22 hr. (19–20 hr.) > 22 hr. (5 hr.)	11 5	4 3
30	1–2 hr. (35–60 min.) > 20 hr. (16–19 hr.)	56 18	12 4	> 20 hr. (16–19 hr.)	15	3	30–60 min. (10–20 min.)	27	6	1–2 hr. (5–20 min.) > 22 hr. (90–120 min.)	20 5	5 2	1–3 hr. (5–50 min.) > 22 hr. (2–3 hr.)	18 7	5 5
25	1 hr. (12–15 min.) 19 hr. (> 6 hr.)	31 4	5 3	> 20 hr. (16–19 hr.)	15	3	18–60 min. (2–12 min.)	15	3	—	—	—	—	—	—
20	1 hr. (5–40 min.)	20	4	> 20 hr. (16–19 hr.) 5 hr. (30–90 min.)	21 5	5 4	30–40 min. (2–12 min.)	19	4	30–60 min. (17–30 min.)	15	3	—	—	—
15	20–40 min. (1 min.)	15	3	1–2 hr. (2–10 min.) 19 hr. (4–60 min.)	11 9	4 4	—	—	—	—	—	—	—	—	—
10	5–15 min. (30–60 sec.)	15	3	3–15 min. (30 sec.)	15	3	3–4 min. (30 sec.)	16	4	2–3 min. (30 sec.)	15	3	—	—	—
5	2–10 min. (30–60 sec.)	15	3	—	—	—	—	—	—	—	—	—	—	—	—

Table 2. *Duration of temporary effects of change*

The effects begin after a time lag of about 1 min. except in those marked*, in which the effects begin within 4 sec. of transfer to the diluted medium.

Percentage of isotonic solution	⅔ frog Ringer						Earthworm saline								
	L. terrestris			*A. chlorotica* (garden)			*L. terrestris*			*A. chlorotica* (garden)			*A. chlorotica* (lake)		
	Duration	Preparations	Worms	Duration	Preparations	Worms	Duration	Preparations	Worms	Duration	Preparations	Worms	Duration	Preparations	Worms
80	No effect	15	3	—	—	—	No effect	15	3	—	—	—	—	—	—
60	—	—	—	—	—	—	No effect	35	5	—	—	—	—	—	—
50	No effect	15	3	No effect	15	3	No effect	40	6	1 min.	15	3	1–2 min.	23	4
40	No effect 2 min.	17 3	4 3	No effect 1–4 min.	10 5	3 3	1–10 min.	35	5	1–1½ min.	15	3	1–1½ min.	16	4
30	No effect 35 sec.	37 37	12 9	1½–5 min.	15	3	20 sec.–2 min.	27	6	45–60 sec.*	25	5	15–80 sec. 15–80 sec.*	15 10	5 3
25	30–90 sec.	35	5	30–90 sec.	15	3	10 sec.–6 min.	15	3	—	—	—	—	—	—
20	55 sec.–15 min.	20	4	30 sec.–12 min.	26	5	2½ min.	19	4	2 min.	15	3	—	—	—
15	1 min.	15	3	30–60 sec.	20	4	—	—	—	—	—	—	—	—	—
10	1 min.	15	3	15 sec.*	15	3	20 sec.	16	4	20–60 sec.*	15	3	—	—	—
5	1 min.	15	3	—	—	—	—	—	—	—	—	—	—	—	—

Table 3. *Duration of ciliary activity in hypertonic media*

Percentage of isotonic solution (E.S.)	*Lumbricus terrestris*			*Allolobophora chlorotica* (garden)		
	Duration	Preparations	Worms	Duration	Preparations	Worms
200	>20 hr.	40	8	5–20 min.	8	3
				>20 hr.	16	4
250	>20 hr.	25	5	6 min.	28	5
				3 hr.	1	1
				>20 hr.	1	1
300	5 min.	23	5	1 min.	15	3
	>20 hr.	7	3			
400	40 sec.	18	3	20 sec.	16	3

Table 4. *Appearance and subsidence of vesicles in hypotonic media*

Percentage of isotonic solution	⅔ frog Ringer								Earthworm saline											
	L. terrestris				*A. chlorotica* (garden)				*L. terrestris*				*A. chlorotica* (garden)				*A. chlorotica* (lake)			
	Time of appearance	Subsidence	Preparations	Worms	Time of appearance	Subsidence	Preparations	Worms	Time of appearance	Subsidence	Preparations	Worms	Time of appearance	Subsidence	Preparations	Worms	Time of appearance	Subsidence	Preparations	Worms
80	No vesicles	.	15	3	.	.	.	.	No vesicles	.	15	3	.	.	.	.	.	.	.	.
60	.	.	.	.	.	.	.	.	1–73 min.	+	18	5	.	.	.	.	.	.	.	.
									1–73 min.	−	17	5								
50	No vesicles	.	15	3	No vesicles	.	15	3	30 sec.–3 min.	−	26	6	No vesicles	.	13	3	No vesicles	.	19	4
									30 sec.–3 min.	+	14	4	20 sec.	+	2	2	1½–3 min	+	4	2
40	30–90 sec.	+	20	4	No vesicles	.	12	3	1–2 min.	−	28	5	45–110 sec.	+	15	3	1–3 min.	+	16	4
					1 min.	+	3	3	1–2 min.	+	7	3								
30	80 sec.–2 min.	+	74	12	No vesicles	.	11	3	30–60 sec.	−	27	6	50–75 sec.	−	20	4	1–2 min.	−	18	5
					1 min.	+	4	3					50–75 sec.	+	5	2	1–2 min.	+	7	3
25	50 sec.–4 min.	−	31	5	No vesicles	.	9	3	25–60 sec.	−	15	3	.	.	.	.	.	.	.	.
	50 sec.–4 min.	+	4	3	3 min.	+	6	3												
20	45–60 sec.	−	20	4	30–60 sec.	+	26	5	35–60 sec.	−	19	4	35–75 sec.	−	15	3	.	.	.	.
15	40–80 sec.	−	15	3	30–60 sec.	−	11	4	.	.	.	.	.	.	.	.	.	.	.	.
					30–60 sec.	+	9	4												
10	35–55 sec.	−	15	3	12–30 sec.	−	15	3	20–35 sec.	−	16	4	10–75 sec.	−	15	3	.	.	.	.
5	45 sec.	−	15	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

preparations which remained active for many hours, the degree of activity was nearly always less than in the controls at 100 %. The cells of *L. terrestris* are rather more resistant to the long-term effects of hypertonicity than are those of *A. chlorotica*.

(ii) *Temporary effects of change*

The typical temporary effect of suddenly transferring preparations from the isotonic to a hypertonic medium was a slowing or complete cessation of ciliary movement. The duration of reduced activity or of inhibition, the time taken for inhibition to become complete, and the time for the first flickering activity after inhibition to reach the final degree of activity, were all extremely variable. In some cases inhibition was not seen at all; in others the period of inhibition was broken by a few minutes of flickering activity. When inhibition occurred, the cilia slowed and stopped within a few minutes.

The variability of the duration of inhibition may be illustrated for the figures for 200 % E.S.; reduced activity or inhibition times at this concentration ranged from 4 min. to 2½ hr. in *L. terrestris* and from 40 min. to 3 hr. in *A. chlorotica*. In these experiments, as in the return to normal from a hypotonic fluid, slowing and inhibition occur when the medium is suddenly changed for a more concentrated one and in this respect the earthworm nephridiostome cilia resemble those of the *Mytilus* gill (Pilgrim, 1953).

(iii) *Return to isotonic solution*

Under natural conditions very little dilution of the coelomic fluid takes place because of the powers of osmoregulation of the worm, but there is no defence against desiccation and the consequent concentration of the coelomic fluid. It is therefore of interest to know whether a period in hypertonic media impairs the ability of the cilia to function when returned to an isotonic medium.

After at least two hours in E.S. after mounting, preparations from *L. terrestris* were transferred to 200 and 300 % E.S. and left for 21 hr. in the case of 200 % E.S. and for between 19½ and 24½ hr. in the case of 300 % E.S. They were then returned to 100 % E.S. and observed.

In all cases ciliary activity began after 8–30 sec. The lip cilia were usually, but not invariably, the first to show slight movement which gradually increased, nearly or quite to the normal degree of activity. The central cell and pre-septal canal cilia burst abruptly into nearly full activity and reached the normal activity level after 1 min. After recovery, full activity was maintained for at least 6 hr.

(3) *The swelling of cells in hypotonic media, and its relation to the temporary acceleration of the beat*

(i) *The formation of vesicles*

During the experiments with moderately hypotonic media (e.g. 40 %) it was observed, in both species, that bubble-like vesicles appeared around the margin of the upper lip of the nephridiostome a minute or so after the change of medium and then gradually subsided. The vesicles began to subside 10–20 min. after their appearance, and took 1–3 hr. to disappear completely. They remained permanently in the greatest dilutions, and were absent in the least (Table 4).

The appearance and subsidence of the vesicles reminds one of the temporary excitation of the cilia produced by a downward salinity change, and the possibility of a relation between the two phenomena was investigated.

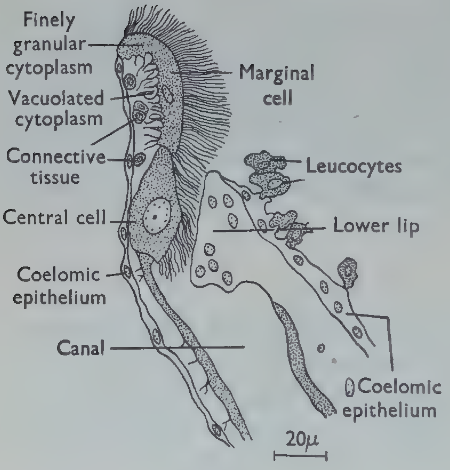


Fig. 2

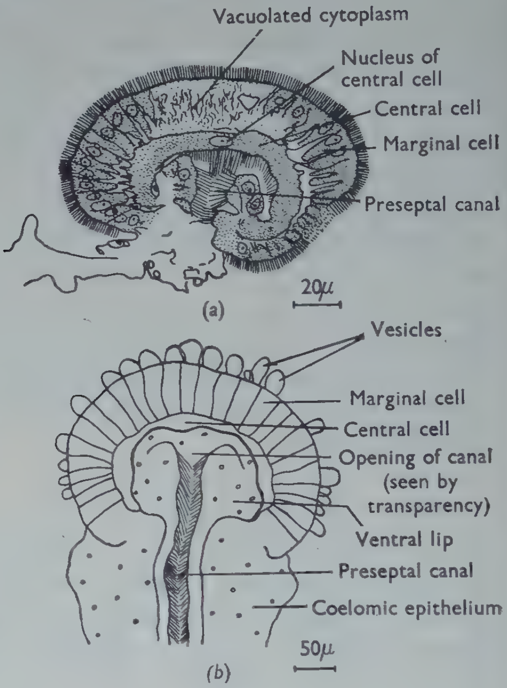


Fig. 3

Fig. 2. Diagram of longitudinal section of nephridiostome of *L. terrestris* to show structure. (After Goodrich.)

Fig. 3. (*a*) Camera-lucida drawing of longitudinal section (at right angles to that shown in Fig. 2) of *L. terrestris* nephridiostome. (*b*) Camera-lucida drawing of a living nephridiostome of *L. terrestris* in 30 % ⅔ F.R.

Very similar blister-like appearances may be seen in cells in the absence of external salinity change—e.g. the bubbling seen in tissue culture cells, and Amoebae when dividing and when exposed to anaesthetics, which seem to be due to syneresis (Goldacre & Lorch, 1950; Goldacre, 1952).

The vesicles in the present experiments appeared to be formed regularly on the marginal cells of the upper lip, usually one to each cell. (The structure of a nephridiostome is shown in Figs. 2 and 3.) Some cells had no vesicles, while others appeared to have several (Fig. 3). Since no cilia were carried out with the membrane of the vesicle, the non-ciliated part of the marginal cells appears to bear the vesicles.

To eliminate the possibility that the vesicles might have been either swollen cells of the coelomic epithelium or connective tissue cells, the site of the vesicles was investigated using *L. terrestris* because of its greater size. Preparations of whole nephridiostomes and sections contributed much evidence but were not conclusive, so Medawar's tryptic digestion method was used (Medawar, 1941).

Isolated nephridiostomes were transferred from $\frac{2}{3}$ F.R. to 0·5 % trypsin in $\frac{2}{3}$ F.R. (pH adjusted to 7·8 with sodium carbonate using phenol red as indicator) and incubated at 30° C. for 1 hr. Then they were transferred to a slide in a drop of the fluid and a cover-slip was placed over them. On tapping the cover-slip with a needle the cells separated from one another. These acted as controls. Others were transferred from $\frac{2}{3}$ F.R. to 25 % F.R. for 5 min., then to 0·5 % trypsin in 25 % $\frac{2}{3}$ F.R. (pH 7·8), and treated in the same way as the controls. These preparations showed beyond doubt that the vesicles are on the marginal cells. One part in 10,000 methylene blue was added to the dissociating fluid and the results recorded by camera-lucida drawings (Fig. 4).

It is possible to vary the salt concentration of a solution without altering the total osmotic pressure, by using dextrose solution as the diluent. If, indeed, the vesicles are due to variations in the salt concentration rather than in the osmotic pressure, the same results should be obtained when the salt concentration is brought down, e.g. to 20 %, while the osmotic pressure is held constant. *L. terrestris* preparations were transferred to $\frac{2}{3}$ F.R. diluted to 25, 20 and 10 % with isotonic dextrose solution. No vesicles appeared in any of these solutions, and there was neither temporary acceleration nor increase in amplitude of the ciliary beat. In 25 % all preparations lasted as long as the controls with the same degree of activity. In 20 % more than half were as good as the controls, the rest failing to survive the night. In 10 % some preparations showed reduced activity after one hour and maintained this for more than 20 hr., in others the cilia stopped beating after about 1 hr. Preparations on being transferred from $\frac{2}{3}$ F.R. to a dextrose solution of the same osmotic pressure lasted for only 40 min. Thus it seems possible that a change in the osmotic concentration is responsible for the temporary effects on the cilia, and the vesicles, whereas the salt concentration is responsible for the long-term effects.

(ii) *Relation of swelling of cells to temporary effects of change*

Experiments were made to study more exactly the time relations between cell swelling and temporary effects on the ciliary beat following a change of medium. It is possible to measure the width of the marginal cells at their widest part with a vernier eyepiece, but it is difficult to be sure of measuring the same cell again, even when the preparation is under constant observation, except in those cases where there are accidental topographical marks. The length of the marginal cells was measured in some preparations, and in others the depth could be seen in optical section and measured.

Sixty-three experiments were made, but complete sets of measurements were obtained for only thirty, because of twisting of the preparations. The vulcanite chamber was used for these experiments so that observations could be continued while the medium was being changed. The complete replacement of the fluid in the chamber was effected in 10 sec. $\frac{2}{3}$ F.R. was used as the isotonic medium and 30% $\frac{2}{3}$ F.R. as the hypotonic medium.

There was a general swelling of the cells before the appearance of the vesicles, which usually continued as the vesicles were formed, but in a few preparations the marginal cells increased in size up to the time of the appearance of the vesicles and then shrank to their original size as the vesicle appeared. In these cases the cell appeared to extrude excess water into the vesicle. After a few minutes the size of the vesicles and of the cells began to decrease again. At maximal swelling the linear dimensions of the cells had increased by 20–30%. In thirteen experiments acceleration of the cilia occurred before swelling, in five experiments swelling

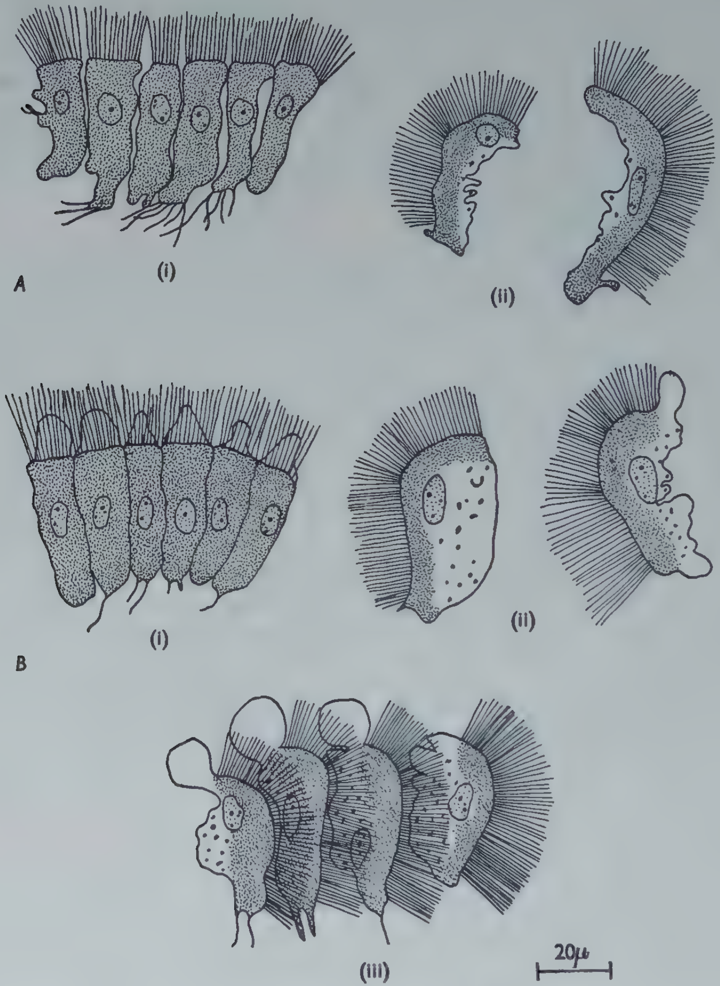


Fig. 4. Dissociated cells of *L. terrestris* nephridiostome. *A*. In isotonic solution: (i) face view, (ii) lateral view. *B*. In 25% $\frac{2}{3}$ F.R.: (i) face view, (ii) and (iii) lateral views.

occurred first, and in five others acceleration and swelling were simultaneous. Of the remaining seven experiments, in one no acceleration occurred and in the other six the measurements were not made at exactly the right moment to be certain which event occurred first. Ciliary accommodation took place either while the cells were still swelling or while the swelling was at a maximum. From these results it may be concluded that variations in ciliary activity are not directly related to the intracellular water content. Wells *et al.* (1940) reached the same conclusion for *Mytilus* gill.

In seventeen experiments the preparations were returned to 100% ⅔ F.R. The activity of the cilia was reduced and the cells shrank and became even smaller than they were originally. This suggests that the regulating process involves ion as well as water transference, which would explain the differences in the effects of dilution with ⅔ F.R. and E.S. As in the change to a more dilute medium, there was no constant time relationship between the shrinking of the cells and reduction of ciliary activity.

(4) *The relative importance of absolute concentration and concentration change*

Pilgrim (1953) found that the ratio final/initial concentration, rather than the final concentration or the arithmetic difference between initial and final concentration, was the effective factor producing the dilution effects in *Mytilus* gill cilia.

An attempt was made to establish whether this is true for the nephridiostome cilia of *L. terrestris* by comparing the effects of changing to a series of low concentrations from 100% and from 150% E.S. The effects are clearly not due to the arithmetic difference between the concentrations, because a change from 100 to 30% (a difference of 70) gives rise to shock effects, whereas a change from 150 to 80% (also a difference of 70) produces no shock effects. As, however, the duration of the shock effect is very variable in earthworms even when the same salinity change is repeated, no further conclusions can be drawn from these results.

(5) *Axial gradients*

Kopenhaver (1937) demonstrated than an axial gradient in the water-absorbing properties of the body wall exists in *L. terrestris* and *A. caliginosa*. Since there was a possibility that such a difference might exist also in the nephridiostomes in *L. terrestris* these were taken from the anterior and the posterior regions of the body and compared. No differences were found between them when they were subjected to a sudden change of medium from 100 to 30% ⅔ F.R.

IV. SUMMARY

1. Suitable media have been found for maintaining the activity of the nephridiostome cilia of two earthworm species for about 24 hr. at 18° C. or below.

2. The nephridiostome cilia of *Lumbricus terrestris* are less resistant to hypotonic media, and more resistant to hypertonic media, than those of *Allolobophora chlorotica*.

3. When the medium is suddenly diluted, the nephridiostome cilia of *L. terrestris* and *A. chlorotica* have a period of hyperactivity and/or increased amplitude of the beat followed by a return to normal activity. The temporary effect after a change to a hypertonic medium takes the form of decreased activity or inhibition.

4. In hypotonic solutions, vesicles are formed on the marginal cells and later disappear provided the dilution has not been too great.

5. A change in the osmotic concentration of the medium is responsible for the vesicles and the temporary effects on the cilia, whereas the inorganic ion concentration is responsible for the long-term effects.

6. There is no axial gradient affecting the activity of the nephridiostome cilia in relation to the osmotic concentration of the medium.

7. No differences were detected between garden and Lake Windermere specimens of *A. chlorotica.*

I wish to thank Prof. G. P. Wells, F.R.S., for his advice, criticism, encouragement and supervision of the investigation; also the Committee of University College, London, for the Margaret Browne Studentship which made the work possible.

REFERENCES

ADOLPH, E. F. (1927). The regulation of volume and concentration in the body fluids of earthworms. *J. Exp. Zool.* **47**, 31–62.

BAHL, K. N. (1945). Studies on the structure, development, and physiology of the nephridia of Oligochaeta. Part VI. The physiology of excretion and the significance of the enteronephric type of nephridial system. *Quart. J. Micr. Sci.* **85**, 343–89.

BAHL, K. N. (1946). Studies on the structure, development, and physiology of the nephridia of Oligochaeta. Part VIII. Biochemical estimations of nutritive and excretory substances in the blood and coelomic fluid of the earthworm and their bearing on the role of the fluids in metabolism. *Quart. J. Micr. Sci.* **87**, 357–71.

ČERNOSVITOV, L. (1945). Oligochaeta from Windermere and the Lake District. *Proc. Zool. Soc. Lond.* **114**, 524–48.

GOLDACRE, R. J. (1952). The action of general anaesthetics on *Amoebae* and the mechanism of the response to touch. *Symp. Soc. Exp. Biol.* no. VI, *Structural Aspects of Cell Physiology*, pp. 128–44.

GOLDACRE, R. J. & LORCH, I. J. (1950). Folding and unfolding of protein molecules in relation to cytoplasmic streaming, amoeboid movement and osmotic work. *Nature, Lond.*, **166**, 497–500.

KOPENHAVER, M. E. (1937). Axial differences in water-absorbing properties of oligochaete tissues. *Physiol. Zoöl.* **10**, 315–26.

MEDAWAR, P. B. (1941). Sheets of pure epidermal epithelium from human skin. *Nature, Lond.*, **148**, 783.

PILGRIM, R. L. C. (1953). Osmotic relations in molluscan contractile tissues. II. Isolated gill preparations from Lamellibranchs (*Mytilus edulis* L., *Ostrea edulis* L., *Anodonta cygnea* L.). *J. Exp. Biol.* **30**, 318–30.

RAMSAY, J. A. (1949*a*). The osmotic relations of the earthworm. *J. Exp. Biol.* **26**, 46–56.

RAMSAY, J. A. (1949*b*). The site of formation of hypotonic urine in the nephridium of *Lumbricus*. *J. Exp. Biol.* **26**, 65–74.

WELLS, G. P., LEDINGHAM, I. C. & GREGORY, M. (1940). Physiological effects of a hypotonic environment. II. Shock effects and accommodation in cilia following sudden salinity change. *J. Exp. Biol.* **17**, 378–85.

THE MOVEMENT OF SEA-URCHIN SPERMATOZOA

By J. GRAY
Department of Zoology, University of Cambridge

(*Received* 20 *May* 1955)

(With Plates 3 and 4)

Although the mechanical activity of spermatozoa plays an essential role in the process of fertilization there is little precise information concerning the type and speed of movement of individual cells in their normal state of activity. This paper represents an attempt to fill this gap with respect to a sea-urchin whose spermatozoa, being of the typical monoflagellate type, provide valuable material for the study of flagellar propulsion in general.

Accurate observation of the movements of fully active spermatozoa encounters three main difficulties: first, the diameter of the tail—on whose activity the propulsion of the head depends—is near the limit of resolution by transmitted light; secondly, the normal frequency of the tail's movements is far above that which can be followed by the human eye; thirdly, direct observation is almost entirely restricted to cells constrained to move in the plane of the microscope's field by the presence of a glass/water or water/air interface. The motion of cells moving freely in a bulk of fluid is extremely difficult to observe. Proximity to a surface not only affects the freedom of the cell to roll or pitch about its own axes, but also exposes the cells to physical conditions different from those in open water.

The difficulty arising from the very small radius of the tail has long been recognized. Fifty years ago Buller (1903) noted that, under normal transmitted light, only the head of an echinoid spermatozoon was visible; he recorded a translatory speed of 120 μ/sec. for the spermatozoa of *Echinus microtuberculatus* by observations on the rate of displacement of the head. More recently the movements of the heads of the spermatozoa of *E. esculentus* and of *Psammechinus miliaris* have been recorded photographically by Rothschild & Swann (1949) using darkground illumination; these records indicate translatory speeds of the order of 190 μ per sec. at 18° C. Apart from the observations of these authors there appear to be few recent observations relevant to the present inquiry.

Some, but not all, of the difficulties of observing the movements of a highly active spermatozoon can be overcome by the use of stroboscopic darkground illumination or by appropriate photographic technique. The results recorded in this paper were obtained by means of a low-power parabolic darkground condenser using a × 20 objective and × 10 ocular. Unless otherwise stated, the observations apply to fresh suspensions of the sperm of *P. miliaris* (P. L. S. Müller; Gmelin) in sea water at about 18° C. when observed on glass slides without cover-slips. The best results were obtained when the slide was previously rinsed in sea water containing a small amount of a wetting agent such as saponin; although, in this way, it is possible to obtain a very thin uniform film yielding good optical contrast, it intensifies such complications as may ensue from closer proximity of the moving sperm to the surface of the slide or water/air interface. As observed in this way, the head of a motionless sperm appears as a triangular* or conical structure about 3 μ long and 1 μ in diameter of base; the outline of the head is brilliantly illuminated. The ovoid or spherical middle piece is also brilliantly illuminated, whilst the tail—less bright than the head and middle-piece—is seen as a straight or sickle-shaped filament 40–45 μ in length (Pl. 1, figs. 1–3); only on very few occasions has a stationary tail exhibited a wave-like form; the characteristic shape of a motionless tail suggests that it possesses some degree of natural elasticity (see p. 794 seq.). The diameter of the tail cannot be much more than 0·3 μ in diameter (Rothschild, 1951); it may end in an extremely fine terminal filament about 5 μ in length.

OBSERVATIONS

(*a*) *Movements of fresh active sperm on the surface of a slide*

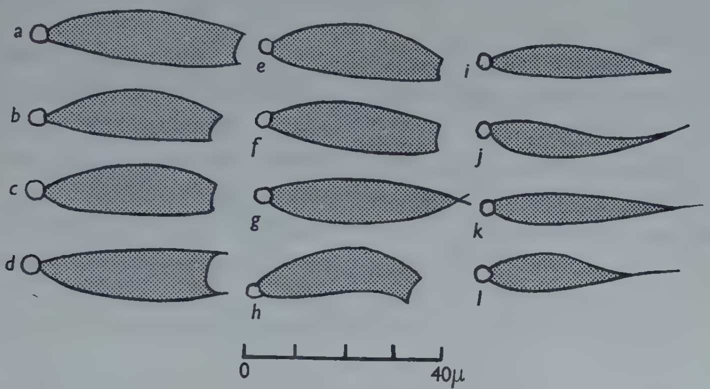
Under darkground illumination an active spermatozoon provides a striking contrast to a sperm at rest. As observed by eye, the moving cell is illustrated diagrammatically by Text-fig. 1. The image of the head is considerably broader than that of a stationary sperm and gives the impression of two images one on each side of the main axis of progression. Behind the head is a less brilliantly illuminated area, variable in shape but, in general, approximating in outline to that of a bullet or of an ellipse. This area is 30–35 μ in length; its breadth varies according to the state of activity of the sperm (see Text-fig. 1 and Pl. 1, figs. 7–14), but in fully active preparations it is of the order of 6–10 μ; it represents the 'optical envelope' of the tail's movements.†

The relationship between the form of the envelope and the activity of the sperm tail is clearly revealed by examination with intermittent light of appropriate intensity and frequency. At 15–18° C. the motion of the tail is 'frozen' at a frequency of illumination of 30–40 per sec.; using flashes of approximately $\frac{1}{500}$ sec. duration (Pl. 1. figs. 4–6), the tail is seen as a brightly illuminated undulatory line, rather more than one wave-length long, and the whole sperm progresses over the field of the microscope without apparent change of form and with only one image of the head visible. If the frequency of illumination is then slightly decreased, waves of curvature pass antero-posteriorly along the tail sufficiently slowly to be followed by the eye. A return to continuous illumination at once yields a return of the characteristic envelopes. The envelope seen under continuous illumination is the result of persistence of vision by the eye of the observer.*

---

* A few observations suggest that the head may be somewhat flattened.

† When viewed by a carbon arc fed by alternating current (or inadequately smoothed direct current) the envelope displays an alternation of light and dark bands similar to those described by Uhlela (1911) for the envelopes of the flagella of some Protozoa. When viewed by an arc fed by direct current of steady voltage the envelope is uniformly illuminated; the banding is therefore an artefact due to pulsations in the intensity of the light source and not, as suggested by Uhlela, to the intrinsic properties of the vibrating flagella. These visual observations have been confirmed photographically. Pl. 1, figs. 10–13, show the bright lines characteristic of illumination from an inadequately smoothed direct current arc; Pl. 1, figs. 7–9, show photographs taken by means of an arc fed by batteries; the broad bands in the latter case are due to the fact that the duration of the exposure was not an exact multiple of the period required for the passage of one complete wave over the tail.



Text-fig. 1. Optical appearance of envelopes of spermatozoa moving over the surface of a glass slide; the head is indicated diagrammatically by a circle. Figs. *a–h* from fresh suspensions; Figs. *i–l* from older suspensions.

(*b*) *The form of the waves generated by the tail*

The visual impression of a wave of changing curvature passing along the tail is an expression of two facts:

(1) That each element of the tail is undergoing a cycle of change in its radius of external curvature each time it sweeps transversely across (or round) the axis of propulsion of the waves (Gray, 1953).

(2) That each element exhibits a phase of bending slightly ahead of that reached by its more posteriorly situated neighbour.

If all the elements constituting the tail bend and execute the whole cycle of their transverse motions in one and the same plane, the envelope of the tail constitutes a plane lamina; whereas, if each element undergoes bending in two planes simultaneously, and these two cycles of deformation are equal in amplitude and always one-quarter of a complete cycle out of phase with each other, the envelope of the tail's motion constitutes a cylinder or cone. Under visual observation, the outline of the envelope might be the same in both cases, but their dynamic properties would differ (Gray, 1953). It is therefore important to know at the outset whether each individual element of the tail executes its movements in a plane, in an ellipse or in a circle; in other words, whether the waves passing along the tail are two- or three-dimensional.

---

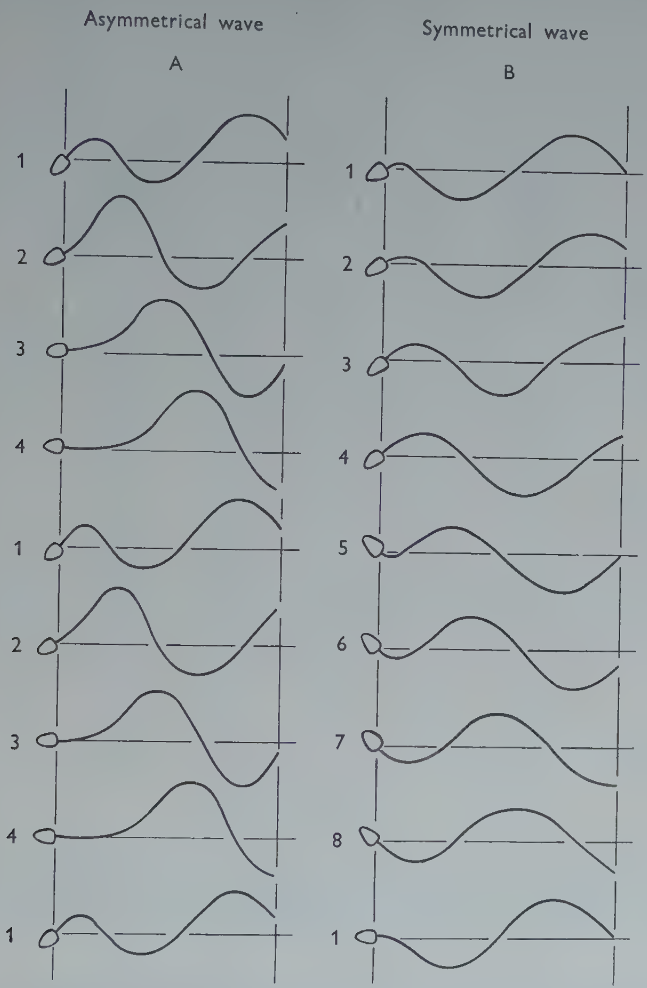
* Persistence of vision occurs whenever the image of an object moves across the retina; it does not occur if the axis of the eye follows the moving object and thereby yields an image stationary with respect to the retina. The visual impression of the tail as an illuminated envelope and the double image of the head are due to the fact that the axis of the eye cannot follow either a point on the tail or the head along an axis normal to the path of the sperm's progression, nor can it follow successive wave crests as they move backwards along this path. On the other hand, the axis of the eye readily follows the head along the axis of progression (since this motion is always in the same direction and is relatively slow), and therefore no persistence of vision of the head occurs along this axis. An interesting demonstration of this principle occurs when the stage of the microscope is abruptly moved; the head then yields a brightly illuminated undulatory track superimposed on the less brilliantly illuminated envelope of the tail, because the image of the head is travelling across the stationary retina; the resultant visual impression is of the same fundamental nature as the tracks recorded on a stationary photographic plate by Rothschild & Swann (1949). (See also Plate II figs. 2–6 of this paper.)

If the plane of observation of a very fresh active suspension of spermatozoa is slightly raised above the level of the slide, individual cells can be observed swimming more or less straight across the field of the microscope. Such cells exhibit one characteristic feature. The visual image of the envelope of the tail alternates rhythmically between the elliptical outline already described and a relatively straight illuminated line (see Pl. 2, fig. 1); the frequency of this change is always very much less than that at which the waves are passing down the tail. Of fourteen cells recorded photographically, the average frequency of this change in form of the envelope was 3 per sec.; the maximum was 6 and the minimum 1·5. The frequency of beat of the tail could not have been less than 24 per sec. It is very difficult to explain these facts on any basis other than that the oscillations of the tail are effectively restricted to a single morphological plane, and that cells, not in contact with an external surface, roll about their longitudinal axes. It does not follow that the contractile fibres responsible for bending are all located in the plane in which the bending occurs.

(c) *Formation and propagation of individual waves*

The development of individual waves at the proximal end of the tail and their subsequent propagation towards the end of the tail can be followed visually by illuminating individual cells at a frequency slightly less than that at which the waves are being generated. Observations of this type indicate that the degree of bending exhibited by an element situated near to the front end of the tail is seldom symmetrical on its two sides. As shown diagrammatically in Text-fig. 2A each wave develops as a transverse displacement of the proximal elements of the tail towards one side of the axis of propulsion. In very few of the cases examined was a transverse displacement of these elements towards one side of the axis followed by one of equal amplitude towards the other (Text-fig. 2B); in other words, the degree of bending exhibited by the two sides of the proximal end of the tail is usually different. These observations are confirmed by the photographic data obtained from spermatozoa where the frequency of the waves was sufficiently low to permit of cinematographic records and where the asymmetry of bending is very marked (see Text-fig. 3).

For quantitative analysis the characteristics of the waves exhibited by a normally active spermatozoon are best determined photographically. Pl. 1, figs. 4–6, are typical of a series of photographs of $\frac{1}{500}$ sec. exposure; these photographs yield images of the tail identical with those recorded by the eye when an active suspension is observed under intermittent light of appropriate frequency with flashes of $\frac{1}{500}$ sec. duration.* As a wave passes away from its point of origin behind the head, its amplitude and wave-length increase whilst its asymmetry usually decreases until

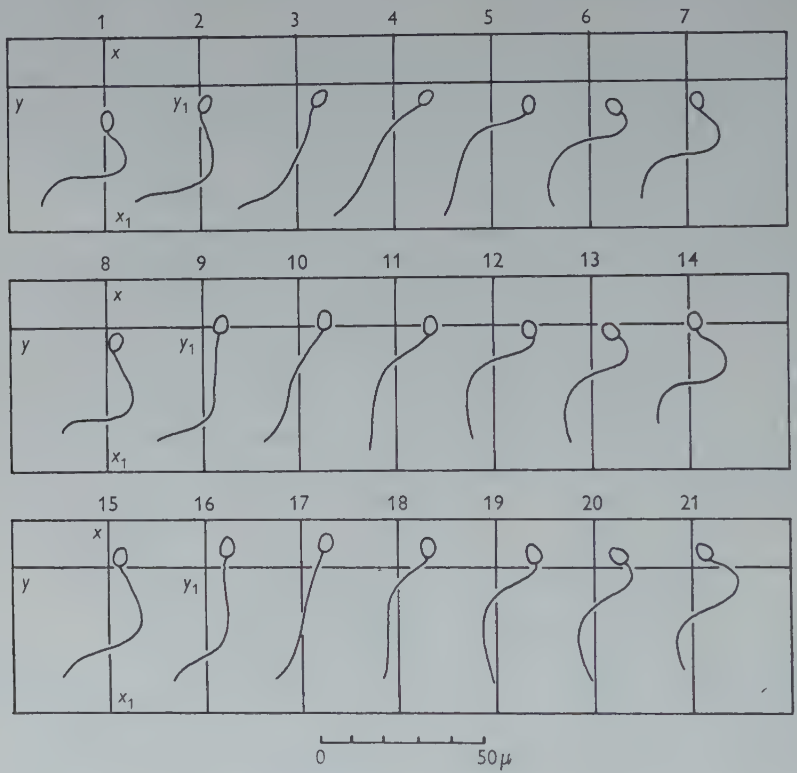


Text-fig. 2. A. Diagram showing asymmetry in the bending on the two sides of the proximal region of the tail. Four phases of bending are shown for each of two waves. Note that the anterior region of the tail only becomes concave on its left side. B. Diagram showing symmetrical bending on the two sides. Eight phases of a single wave are shown.

the wave crest has reached a point about half-way along the tail. At this phase the form of the waves varies in different samples of spermatozoa, but is relatively constant during the early life of any given suspension. Table 1 refers to a suspension in which the average wave-length (λ) was $24\,\mu$ and the average amplitude (b) was $4\,\mu$, giving an average value of $b/\lambda = 0{\cdot}17$. In some instances the form of the wave conforms very closely to that of a sine curve (see Text-fig. 4); in others, the asymmetry characteristic of the proximal region is observable over the whole length of the tail.

---

* The intensity of light required to yield an adequate photographic image of the tail causes extensive scatter from the surface of the head; the latter appears in these photographs as a relatively large circle of light. This difficulty does not arise in visual observations with lower intensity of illumination.

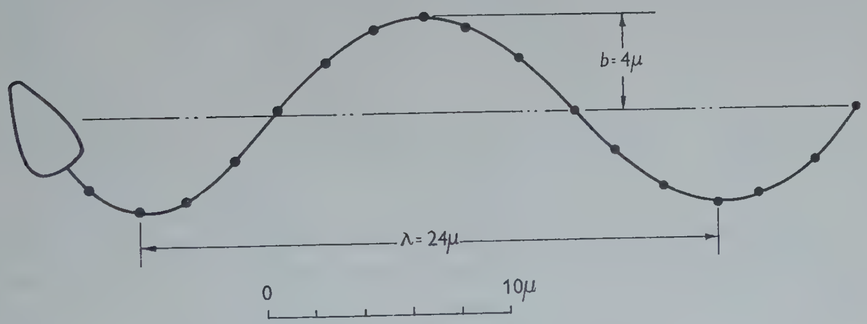


Text-fig. 3. Tracings from a cinematograph record (twelve frames a second) of a spermatozoon propagating three waves in 1·75 sec. Note the asymmetry of bending: the transverse grid-line yy_1 and the vertical grid-line xx_1 is the same in all the figures; the head advances when the tail straightens out (e.g. phases 1–4) and moves backward during the bending phases (phases 4–7).

From a dynamical standpoint it is useful to have a quantitative estimate of the amount of deformation undergone by each element of the tail during a complete cycle of its movements. An element is bent to its maximum extent when it is situated at the crest of a wave; but, as the amount of bending changes relatively rapidly along the curve, only very rough values are available by direct measurements of curvature; useful estimates can be derived from waves of regular form. The radius of curvature at the crest of a sine wave approximates to $\lambda^2/4\pi^2 b$, and therefore the radius of maximum curvature of each side of each element of the tail in Text-fig. 4 changes from $+4$ to $-4\,\mu$ during each complete contractile cycle. As the radius of the tail is less than $0{\cdot}2\,\mu$ the difference in length between the two sides of a fully bent element is not more than 10% of the length of a central neutral axis. A laterally situated contractile fibre 1 μ in length (when neither contracted nor stretched) would shorten to 0·95 μ when fully shortened and extend to 1·05 μ when fully stretched. The process of shortening occupies one-quarter of the whole cycle, and consequently for a wave frequency of 33 per sec. the rate of shortening or elongation would be of the order of 6–7 μ per sec. Both the total degree of shortening of each tensile element and the rate at which this occurs are thus quite small.

Table 1

Sperm no.	Length of envelope in μ	Wave-length (λ) in μ	Amplitude (b) in μ	Total length of tail in μ	Ratio b/λ
1	35	23	4	45	0·174
2	33	24	4	38	0·167
3	34	27	3·5	42	0·130
4	35	27	4	42	0·148
5	35	23	4	40	0·174
6	34	21	4	42	0·190
7	32	24	3·5	42	0·146
8	34	24	4	42	0·167
9	32	22	3·8	40	0·170
10	33	24	3·8	40	0·156
11	32	23	4	38	0·174
12	35	29	5	40	0·172
13	29	24	5	40	0·209
14	30	21	3·2	40	0·160
Average	33	24	4	41	0·167



Text-fig. 4. The outline of the tail was derived from a photograph $\frac{1}{500}$ sec.) of an active spermatozoon; the points are derived from a sine curve of amplitude 4 μ and wave-length 24 μ.

The duration of full activity of spermatozoa in a dilute suspension is limited to a few minutes. Towards the end of this period a marked change frequently occurs in the form of the tail's envelope (see Text-fig 1, *i–l* and Pl. 1, figs. 14–17); its width decreases progressively from the anterior to the posterior end until only a short region behind the middle piece and head remains active; finally, the whole tail becomes motionless. This progressive reduction in the amplitude of the waves as they pass along the tail is closely comparable to the damping effect of external viscosity on waves passively generated in an elastic rod (see p. 794 seq.), and strongly suggests that the normal movements of distal elements of the tail can only be sustained by energy generated by the elements themselves and are not the mechanical result of movement executed by more proximally situated elements (see p. 794 seq.).

(*d*) *Frequency and rate of propagation of waves*

The average frequency of the waves as determined stroboscopically for the cells concerned in Table 1 was 33 per sec., and since the wave-length was 24 μ the average speed of conduction of the wave (as measured along their axis of propagation) was 800 μ per sec. The average speed of forward movement of the whole sperm was, for these particular cells, 180 μ per sec., giving a ratio of propulsive speed to wave speed of 0·23. An alternative method of estimating the frequency of the waves is available by counting the number of undulations exhibited by photographs of the track traced out by the head in a known time, since each undulation represents the passage of one wave along the tail (see p. 783). A series of such observations, the average frequency for which was 36·9 per sec., is shewn in Table 2; the photographs published by Rothschild & Swann (1949) indicate frequencies of 30–40 per sec. Over a considerable range of frequencies the head travels forward between 5 and 6 μ during each wave, so that a propulsive speed of 200 μ per sec. indicates wave frequencies between 33 and 40 per sec.; this figure agrees with the stroboscopic determinations.

Table 2

Frequency of waves per sec.	20–24	25–29	30–34	35–39	40–44	45–49	50–54	36·9
No. of cells	0	2	7	11	6	3	0	Arith. mean

For waves 24 μ in length, frequencies varying from 33 to 40 per sec. would be equivalent to velocities of propagation of the waves relative to the head of 800–1000 μ per sec., according to the frequency of propagation. The ratio of the speed of wave propagation to the speed at which the spermatozoon propels itself through the water is of fundamental importance in an analysis of the dynamical principles involved during the propulsion of the cell; this aspect of the problem will be considered in a separate paper (Gray & Hancock, 1955).

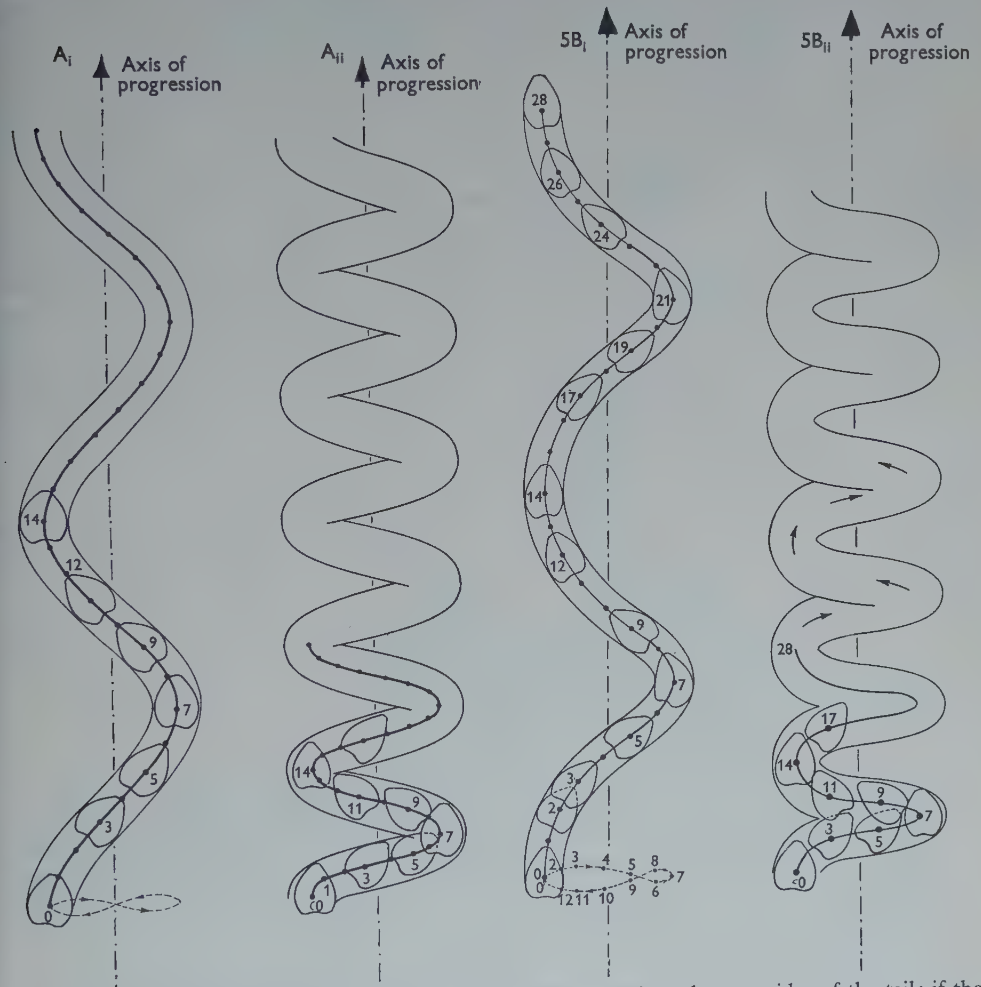
At present, little information is available concerning the frequency with which waves are generated by spermatozoa approaching the end of their active life. Two types of change in wave frequency have been observed:

(i) Cells which stop abruptly after a phase of full activity; in some cases the cessation of movement appears to be permanent, in others active movement is suddenly resumed.

(ii) Cells in which the frequency of the tail's movement gradually decreases without any very drastic change in the form of the waves. The characteristic features of one of these slowly moving cells are illustrated by Fig. 3, the amplitude of the waves being somewhat larger than that of fully active cells.

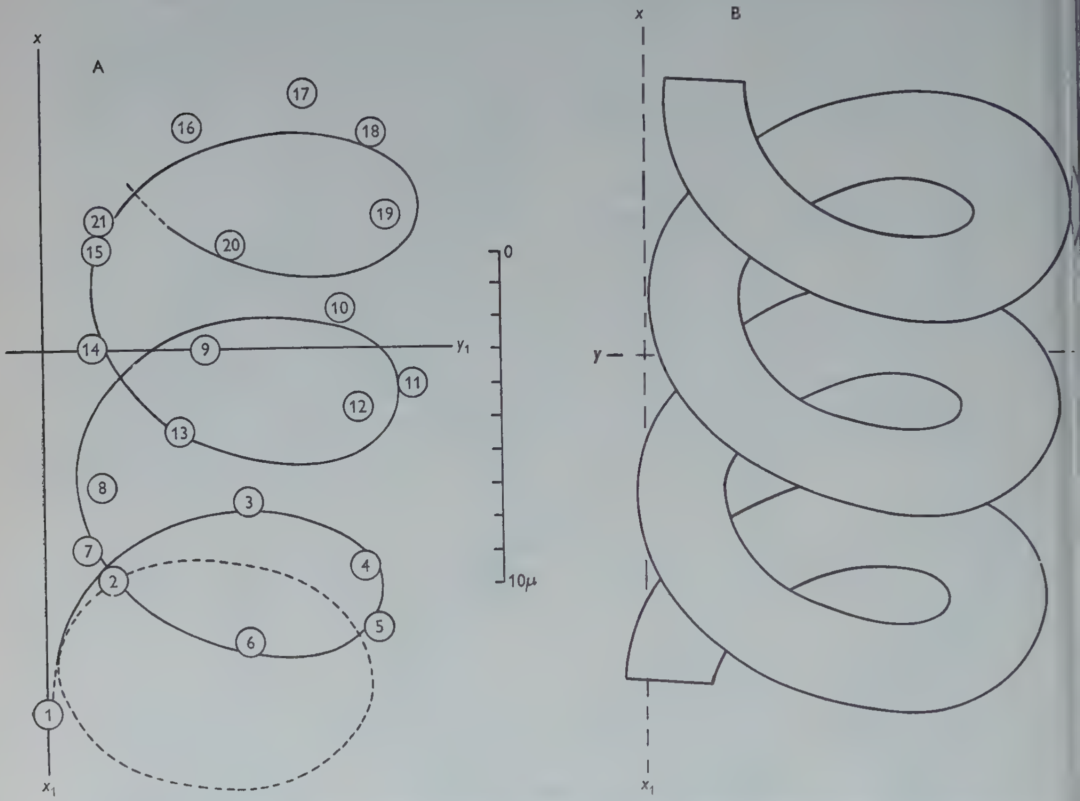
(*e*) *The path of movement of the head*

As noted by Rothschild & Swann (1949), and seen in Pl. 2 of the present paper, the track of the head of a spermatozoon is by no means always symmetrical about the main axis of progression; the rate of forward movement of the head is usually greater when the head is displaced to one side of the axis of progression than to the



Text-fig. 5. In Text-fig. 5A$_{\text{i}}$ the bending wave is symmetrical on the two sides of the tail; if the spermatozoon were imagined to slide forward within a closely fitting rigid tube the head would move along a symmetrically undulating track (shown by the dark line running through the axis of the head) at a speed equal to that at which the waves were propagated backward over the tail. When a spermatozoon is moving in a fluid medium the forward speed is very much less than the speed of propagation of the waves, and the head follows the dark line shown in Text-fig. 5A$_{\text{ii}}$—this line being symmetrical about the axis of propulsion. Text-fig. 5B is similar to Text-fig. 5A except for an asymmetry of curvature on the two sides of the body—note the asymmetry of the head track in Text-fig. 5B$_{\text{ii}}$. In Text-figs. 5A$_{\text{i}}$ and 5B$_{\text{i}}$, the dotted line at the point 0 shows the approximate movement of the head if the forward speed of propulsion were zero.

other.* If the waves developed at the front end of the tail were symmetrical about their axis of propagation, the head of the moving sperm would exhibit a symmetrical figure of eight displacement relative to axes passing through the centre of gravity of the sperm, and a symmetrical sinuous track relative to fixed axes (Text-fig. 5A). If, on the other hand, the waves are asymmetrical, the track of the head must also be asymmetrical (see Text-fig. 5B). Text-fig. 6 shows the relationship between the track of the head and the passage of an individual wave derived from the cinematograph record of the slowly moving sperm shown in Text-fig. 3. As a wave forms at the proximal end of the tail, the head may even move backwards relative to the ground; as the wave subsides the head moves forward at its maximum speed.

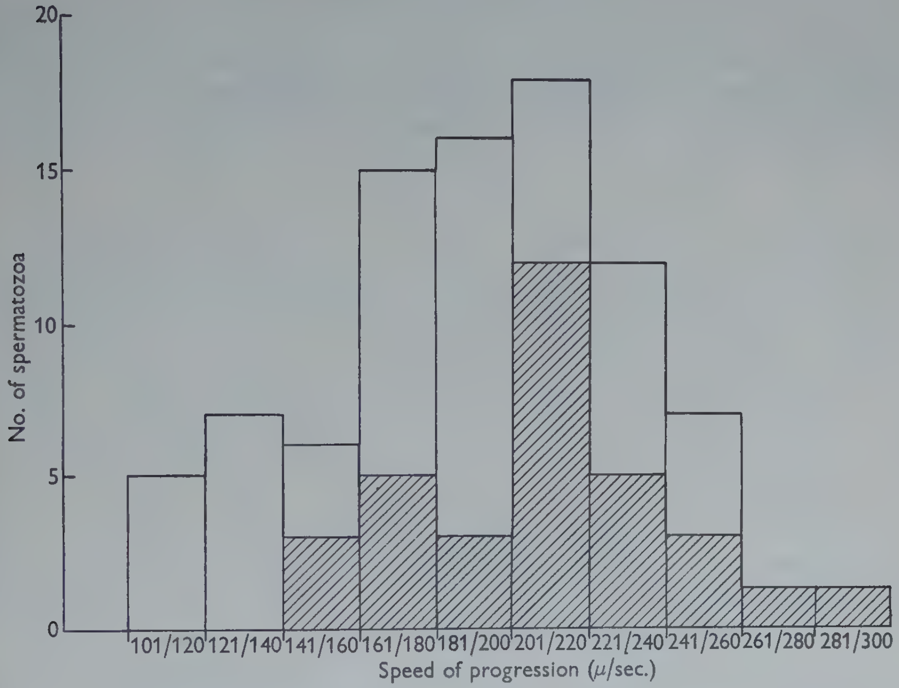


Text-fig. 6. A. Track of the central point of the head of the spermatozoon shown in Text-fig. 3; the grid lines xx_1 and yy_1 are the same in both figures. Successive positions of the head in Text-fig. 6 are shown by numbered cicles and correspond to the numbers in Text-Fig. 3. The thick continuous line shows the track which would have been followed had the head moved along the surface of the dotted ellipse and also moved forward at a constant speed of about 6 μ during the passage of each wave (i.e. in about $\frac{1}{2}$ sec.). B. Diagrammatic illustration of the illuminated head track of Text-fig. 6A. Compare with Pl. 2, figs. 2–6.

* The asymmetry of the head tracks was interpreted by Rothschild (1951) as an oblique projection of a regular helix resulting from three-dimensional waves passing along the tail. The axis of microscopic vision is, however, so nearly normal to the plane of observation as to render the microscopic projection of a regular helix indistinguishable from a two-dimensional sine wave.

(*f*) *Speed of translatory movement*

The average speed of translation of the sperm of *P. miliaris* as calculated by Rothschild & Swann (1949) from the length of the head track during $\frac{1}{4}$ or $\frac{1}{2}$ sec. was 190 μ per sec. at about 18° C. Data obtained in this way from thirty-three cells during the present investigation yielded an average speed of 208·5 μ per sec. for an average wave frequency of 36·9. In another suspension the average translatory speed for fifty-six cells (as determined cinematographically) was 181 μ per sec., for an average wave frequency (as determined stroboscopically) of 33 per sec. The average speed of the eighty-nine cells was 191·4 μ for an average wave frequency of 34·5 per sec., or 5·5 μ per wave.



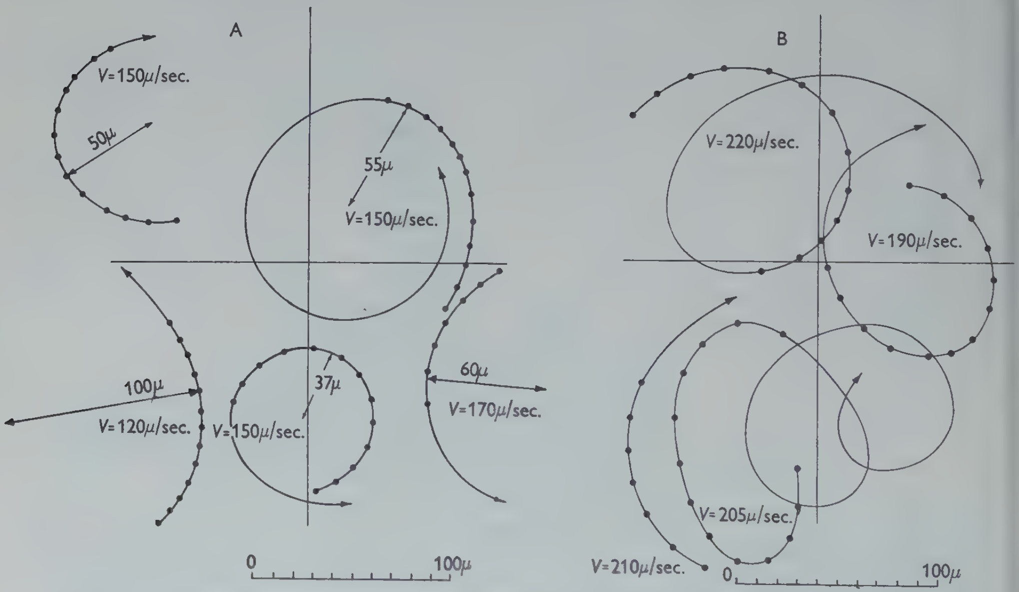
Text-fig. 7. Histogram of variation in speed of progression. The hatched areas indicate observations derived from the length of head tracks traversed in $\frac{1}{2}$ or $\frac{1}{4}$ sec.; the clear areas are from cinematograph records.

The scatter of translatory speeds observed in the eighty-nine cells referred to above (see Text-fig. 7) is presumably due to two main factors—variation in wave form and variation in wave frequency. As the width of the tail's envelope decreases with increasing age of the suspension the translatory speed falls, the average translatory speed of cells with envelopes of the form shown in Text-fig. 1, *i–l*, and Pl. 1, figs. 15–17, was of the order of 50 μ per sec. Unfortunately, no data are available concerning the frequency of the waves generated by these cells, but the drop in speed appears to be greater than that which can be accounted for by change of wave form alone.

All the above records apply to spermatozoa moving over the surface of a glass slide. Reliable records of cells moving freely in a bulk of liquid are very difficult to obtain, but among fourteen cells free to roll about their longitudinal axes (see p. 778) the maximum propulsive speed was 204 μ per sec., and the minimum 130 μ per sec.; these speeds do not differ greatly from those of cells moving closer to a surface.

(*g*) *Direction of translatory movement*

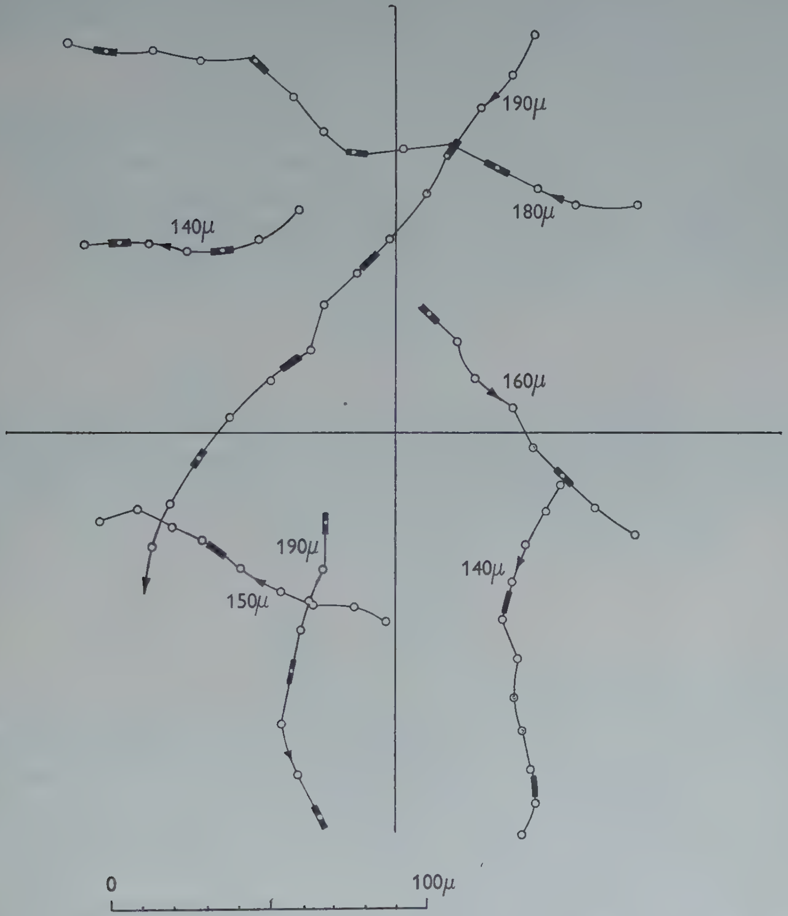
Very few of the cells moving over the surface of a slide swim along a straight line; the great majority travel along a curved track whose radius of curvature varies considerably from one spermatozoon to another (Buller, 1903); in *P. miliaris* the radius usually varies from 30 to 100 μ (Text-fig. 8). In some cases, the track of the sperm is almost circular, a complete circle being effected in 1·0–3·0 sec.; in other cases, the radius of curvature varies and the resultant track forms a flat spiral; in other instances the radius of curvature varies more irregularly and may occasionally change in sign. No obvious relationship appears to exist between degree of curvature of the track and speed of translation along the track. In contradistinction to a cell moving over the surface of the slide (with its tail beating in a plane parallel to this surface) a cell which rolls about the median longitudinal axis of its envelope



Text-fig. 8. A. Head tracks (from cinematograph records) of five cells moving over the upper surface of a slide and viewed from above, the radius of yaw varying from 37 to 100 μ. Four out of the five cells are rotating counter-clockwise. The dots indicate the position of the head at $\frac{1}{12}$ sec. intervals. B. Head tracks of four cells moving over the top surface of a relatively deep drop—also viewed from above: all four cells are moving clockwise.

has a relatively straight track (Text-fig. 9). The effect of a roll on the path of progression of a yawing cell is to transform this path from the arc of a circle to a helix.

For many purposes it is necessary to describe the movements of a spermatozoon relative to three internal morphological axes. The first of these (xx_1) is provided by the median longitudinal axis of the head; the second (yy_1) can be arbitrarily fixed by a line normal to xx_1 and lying in the plane of the tail's envelope. If yy_1 is drawn through the head of the sperm at any point O on the xx_1 axis, the third axis (zz_1) is provided by a line normal to xx_1 and yy_1; in this way four points $(y, y_1; z, z_1)$ are fixed on a cross-section of the head through O.



Text-fig. 9. Track of head (from cinematograph records) of spermatozoa rolling about their longitudinal axes. The lines are thickened at positions where the envelope of the tail appears edge-on in the record. The dots indicate positions at $\frac{1}{12}$ sec. intervals. The speed of progression is shown in μ per second.

Strictly speaking the movements of the sperm should be defined in terms of the three axes xx_1, yy_1 and zz_1. Rotation of the whole sperm about xx_1 constitutes a *roll*, rotation about yy_1 a *pitch*, and rotation about zz_1 denotes *yaw*. For most purposes, however, reference to xx_1, yy_1 or zz_1 proves cumbersome, and it is convenient to replace it by an arbitrary, but precise, use of the terms 'dorsal', 'ventral', 'right' and 'left'. If the point z on the surface of the head be denoted as 'dorsal', then z_1 is 'ventral', y is 'left' and y_1 is 'right'.

A *roll* about the axis xx_1 can therefore be either towards the 'right' (y_1) side or towards the 'left' (y) side; similarly, a yaw about zz_1 can either be towards the 'right' (y_1) or towards the 'left' (y); if this nomenclature is adopted it is essential to bear in mind that the terms dorsal, ventral, right and left refer to the internal morphological axes of the spermatozoon and not to axes which refer to an external environment or observer.

If the forces propelling a spermatozoon drive the cell forward along the longitudinal axis of the tail's envelope and, at the same time, cause this axis to yaw steadily to one side, the head of the sperm travels along the arc of a circle whose radius (ρ) depends on the velocity of the sperm's progression (V) and on the frequency per second (ϕ) with which the cell yaws through 360°:

$$\rho=\frac{V}{2\pi\phi}$$

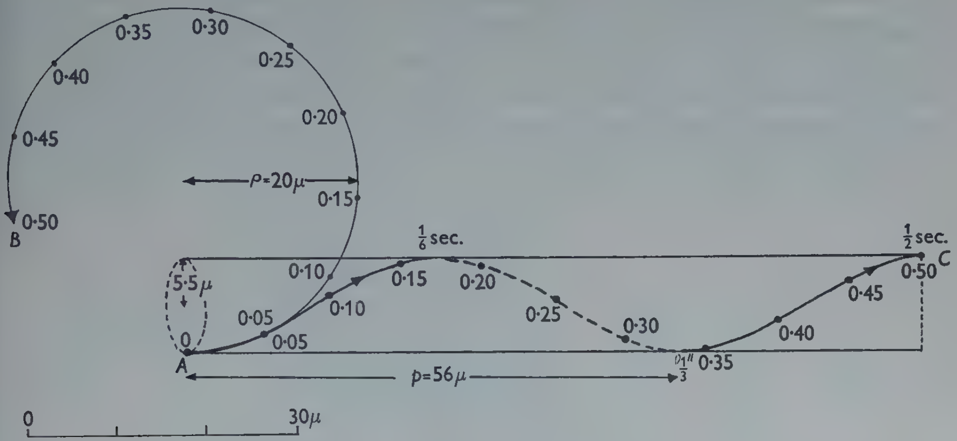
If, in addition to a steady yaw, the spermatozoon also rolls about its longitudinal axis n times per sec., the length of track (V/n) traversed during each complete cycle of roll is transformed from a plane arc into one complete turn of a regular helix. The radius (r) and the pitch (p) of the helix depend on the values of V, n and ϕ:

$$r=\frac{V^2}{4\pi^2n^2\rho}=\frac{V\phi}{2\pi n^2},$$

$$p=\left[\left(\frac{V}{n}\right)^2-4\pi^2r^2\right]^{\frac{1}{2}}.$$

If a spermatozoon travelling in a plane along an arc of a circle 75 μ in radius at a translatory speed of 200 μ per sec. begins to roll twice a second, the resultant helical path of progression has a radius of about 3·3 μ and a pitch of nearly 100 μ. So long as the cell rolls about its longitudinal axis, the axis of progression is a straight line, and the more frequently it rolls the less is the cell's displacement (r) from this line. For observed values of V, n and ϕ the pitch of the helix is large compared with its radius. The greater the rate of yaw the greater is the frequency with which the cell must roll in order to maintain the same radius of helical progression. From a functional standpoint the ability to roll provides the spermatozoon with a mechanism for maintaining a rectilinear axis of progression even when the forces operating in a 'lateral' plane tend to induce a very pronounced and persistent yaw.

When a spermatozoon is moving forward with both yaw and roll its motion (relative to the surrounding medium) is essentially that of a lamina moving over a 'screw' surface, each element of the tail sweeping 'transversely' across this surface much more frequently than the cell, as a whole, moves along one complete turn of the screw. A rolling cell which progresses without yaw follows a straight line, but its envelope continues to lie on the surface of a screw whose width is the width of the envelope and whose pitch depends on the forward speed and frequency of roll. The envelope can be regarded as a strip slightly twisted about its long axis. The effect of yaw and roll on the path of the sperm is illustrated by Text-fig. 10.



Text-fig. 10. Diagram showing the effect of rolling on the path of progression of a spermatozoon whose path of motion is otherwise the arc of a circle of radius 20 μ and whose forward speed is 200 μ per sec.; the spermatozoon traverses the arc *AB* in ½ sec., the points on the arc indicate the position of the head at 0·05 sec. intervals. If the spermatozoon rolls 'anti-clockwise' through 360° three times a second, the path of progression becomes a helix *AC* of approximate radius 5·5 μ and pitch 56 μ; the path of the spermatozoon describes 1·5 turns of the helix in 0·5 sec. The diagram does not show that the plane of the arc *AB* is inclined at an angle to the axis of the helix. (See Text-fig. 11.)

(*h*) *Apparent predominance of anticlockwise yaw*

As recorded many years ago (Dewitz, 1886), the spermatozoa of insects show a marked tendency to move in an anticlockwise direction when viewed towards the surface over which they move. This remarkable phenomenon, also observed in *Sphaerechinus granularis* sperm by Buller (1903), has been noted during the present work on *P. miliaris*. In a relatively deep suspension, the spermatozoa show a marked tendency to concentrate at, and swim over, either the surface of the slide or the upper (water/air) surface of the drop. Table 3 shows the direction of curvature of their movement as derived from cinematograph records.

Although an asymmetry of propulsive forces on the two sides of the tail may well be the result of an asymmetry in the form of the waves, this does not explain why a large proportion of the cells move in an anticlockwise direction when viewed against the surface of the slide. Buller suggested that the spermatozoa of sea urchins should be regarded as essentially bi-laterally symmetrical systems, possessing morphological definable right, left, dorsal and ventral surfaces, and that they applied the same morphological surface to any external surface with which they came into contact. A simpler picture can, however, be derived from the fact that the motion of a majority of spermatozoa involves yawing and rolling components.

Relative to its own internal morphological axes (see p. 787), a yawing cell has two degrees of freedom; it can yaw either towards its right side or towards its left. Similarly, the cell can roll either towards its right side or its left; in the first case the direction of roll is clockwise relative to the cell's own internal axes and to an observer viewing the cell from its posterior end; in the second case the roll appears anticlockwise relative to the same observer. The problem is to define the circumstances under which 80 % of the cells will appear, under the microscope, to yaw over the upper surface of a slide in an anticlockwise direction relative to the observer. As already explained the path of progression followed by the head of a rolling and yawing spermatozoon is a helix. This helix can be either right-handed or left-handed; it is right-handed if the cell is rolling to the right, i.e. in a clockwise direction relative to an observer viewing the cell from its posterior end; the helix is left-handed if the direction of roll, relative to such an observer, is anticlockwise; the right-handedness or left-handedness of the helix does not depend on whether the cell is yawing towards or away from its own right surface. These principles can be visualized from Text-fig. 11 in which the helix of progression is traced on the surface of a cylinder. If the orientation of the sperm relative to the helix is such that its right side lies on the helix whilst its left side is away from the surface of the circumscribed cylinder, (i.e. the envelope is lying on the screw surface seen in Fig. 11 with its right side in contact with the surface of the cylinder) the sperm must be yawing towards its right side; if the left side is toward the cylinder the sperm is yawing to its left side. Relative to an observer viewing the cell from its posterior end, however, the direction of roll is always anticlockwise for a left-handed helix and clockwise for a right-handed helix; relative to an observer viewing the cell from its anterior end the apparent direction of roll is, in both cases, reversed, e.g. a cell advancing toward the observer along a left-handed helix appears to be rolling clockwise. If for any reason a cell is prevented from rolling its path becomes restricted to the surface of a plane and the cell will, relative to the observer, yaw over this surface in the same direction as that in which it was originally rolling; if the original roll was anticlockwise relative to the observer, the direction of yaw on the plane surface will also be anticlockwise

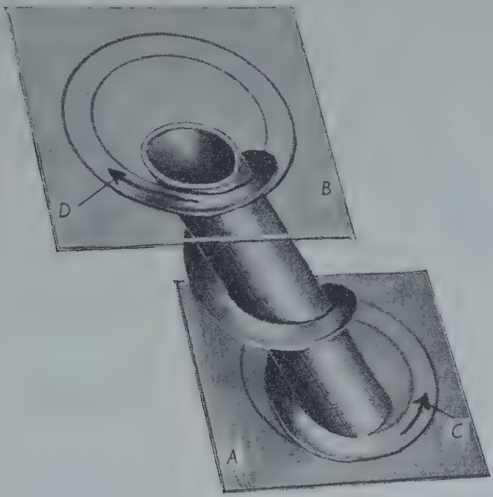
Table 3

Sperm on upper surface of slide (viewed from above)				Sperm at upper surface of drop (water/air interface) (viewed from above)			
Suspension	Clockwise	Anticlock	Doubtful	Suspension	Clockwise	Anticlock	Doubtful
A	7	56	7	A	51	6	3
A	2	33	4	A	35	3	9
A	3	26	6	A	26	3	6
A	1	6	0	A	54	7	14
B	0	8	0	B	7	0	0
C	6	29	9	C	43	4	8
Total	19	158	26	Total	216	23	40
%	9·3	77·8	12·8	%	77·4	8·2	14·3

relative to the observer. The fact that 80% of the cells conform to this rule is thus explicable on three relatively simple assumptions:

(i) That the forces exerted by the medium on the large majority of cells causes them to yaw towards the right or left side of their own median longitudinal axis, the relative numbers yawing in the two directions being immaterial.

(ii) That the reaction from the water, in at least four cells out of five, forces the cell to roll in an anticlockwise direction relative to an observer looking along the axis of progression in the direction of movement; in other words, in open water the motion of the cell is equivalent to that of a left-handed screw.



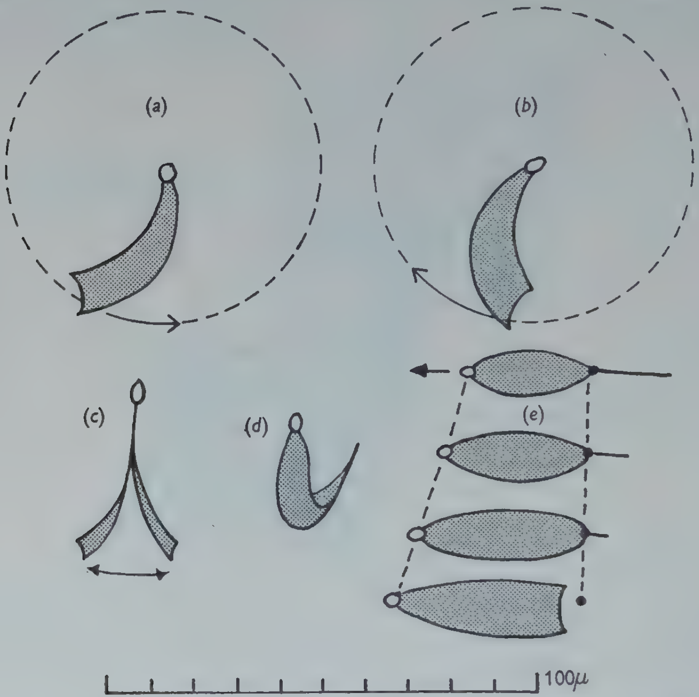
Text-fig. 11. The diagram shows a left-handed screw surface projecting from the surface of a circular cylinder, and terminating smoothly on two plane surfaces *A* (slide) and *B* ('cover-slip'). Spermatozoa may be visualized with the plane of their envelopes lying on the screw surface, and moving up or down this surface. All spermatozoa moving down the surface must be rolling anticlockwise relative to an observer viewing them from *B* to *A*; if, on reaching the surface *A*, the cells cease to roll, they will proceed to yaw over this surface in the direction of the arrow *C*, i.e. in an anticlockwise direction. All spermatozoa moving up the screw surface must roll clockwise relative to the observer, and will yaw clockwise over the surface of *B* in the direction of the arrow *D*.

(iii) That close proximity to a glass or other plane surface prevents the cell from rolling. Four cells out of five will, relative to the observer, move anticlockwise over the surface of the slide, irrespective of whether they are yawing towards their own right or left sides. The apparent direction is reversed relative to an observer looking at a coverslip towards whose under surface a cell is approaching or has come into contact (see Text-fig. 11).

Until data are available concerning the relationship of yawing and rolling frequencies to the frequency of the propulsive waves passing down the tail, it is difficult to say how far the marked tendency to behave as a left-handed screw is the result of static structural asymmetry of the head, and how far to the dynamic effect of a slight left-handed twist in the envelope of the tail's movements.

(*i*) *Behaviour of sperm when attached to a glass surface*

Within a few minutes of placing a drop of a suspension on a slide, an increasing number of sperm become attached to the glass surface by their heads although their tails continue to vibrate actively. In nearly every case, the envelope of the tail yaws round the point of attachment of the head with a frequency of about two or three times per second. The tail envelopes of these sperm differ from those swimming freely over the surface of the slide in that their 'leading' surfaces are more markedly convex and their 'trailing' surfaces more concave (see Text-fig. 12); if, as occasionally happens, the direction of yaw is reversed, the curvature of the surface is also reversed. There can be little doubt that the yawing movement about a fixed head is an extreme case of the yawing phenomenon already described for the majority of cells moving over the surface of the slide.



Text-fig. 12. *a*. Spermatozoon on glass surface rotating anticlockwise about the head. *b*. Similar cell rotating clockwise. Note the convex 'leading' surface. Plane of envelope is parallel to slide. *c*. Spermatozoon attached by head, with plane of envelope vertical at proximal end and twisted at distal end. The whole cell oscillated in the direction of the arrows—the distal end untwisting at the reversal of the direction of movement. *d*. Twisted envelope. *e*. Inhibition of bending waves by restraint from a foreign particle (shown by the dot) attached to the slide.

In the large majority of cases the plane of the envelope of a fixed sperm is parallel to that of the surface to which the head is attached, but a few cases have been observed when the plane of vibration of the tail is oblique or normal to the slide. Attachments of this kind are not only comparatively rare, but tend to be transitory; they are therefore difficult to record photographically. On four occasions visual observations were made of cases in which the plane of vibration of the tail was effectively normal to the plane of the slide; the form and motion of the envelope were then comparable to those of a thin elastic lamina (viewed edgewise) vibrating from side to side through an arc of about 30°. The envelope resembled a brightly illuminated bent and twisted blade with its leading surface convex and its trailing surface concave. The posterior region of the envelope from time to time widened considerably, giving very clearly the impression of a twisted blade. Whilst undue weight should not be placed on phenomena which are relatively seldom observed, it will be noted that the behaviour of the sperm described above conforms with the conclusion that the envelope of the tail's movements is essentially a lamina whose surface may be bent or twisted under appropriate circumstances.

Occasionally a sperm may be attached to the slide by the tip of its tail, leaving the head free to move over the surface; in such cases the envelope tapers posteriorly towards the point of attachment round which the whole sperm rotates. An interesting modification of this phenomenon was seen on one occasion when the sperm tail was attached at a point about one-half of its length behind the head; when first observed the region of the tail proximal to the point of restraint was actively beating and giving a well-defined envelope; the region distal to the point of attachment remained stationary. The whole sperm moved slowly forwards—the region proximal to the restraint gradually increasing in length and the envelope increasing accordingly. It would appear that waves cannot pass a region of mechanical restraint (see p. 797).

The general impression derived from the observation of an ageing suspension is that the versatility of the tail's movements should not be underestimated, particularly when any part of the cell is subjected to any form of external mechanical restraint.

DISCUSSION

The rhythmical changes in form executed by the tail of an echinoid spermatozoon are fundamentally the same as those of most, if not of all, other undulatory organisms. Some or all of the successive regions of the body can, by active internal effort, induce a change in the radius of their external curvature and, during this process of bending, apply equal but opposite bending couples to the elements lying distally and proximally to itself; provided the phase of bending reached by an element differs by an appropriate amount from that of its neighbour, waves of changing curvature pass along the body and propulsion ensues (Gray, 1953). Two main problems require investigation—the nature of the internal bending mechanism and the nature of the system which maintains the difference in phase between adjacent segments.

Bradfield (1953) and more recently Afzelius (1955) have shown that the tail of a *Psammechinus* spermatozoon contains nine fibrils radially arranged within the perimeter of a cylindrical membrane and two finer fibrils lying along the axis of this cylinder. Whilst any attempt to assess the functional significance of these structures must be regarded as highly speculative, it is perhaps permissible to consider certain general principles which may eventually have to be taken into consideration. In order that any short element of the tail should be capable of active bending it must contain contractile elements and at the same time be capable of resisting compression. It is impossible to say whether the radial fibrils are contractile and the matrix or membrane of the tail able to resist compression or vice versa. For the time being it seems not unreasonable to regard the nine radially arranged fibrils as contractile, in the sense that when excited by a stimulus they shorten in length and develop tension against the resistance of the compression elements present in the tail and against external resistances. From this point of view it is useful to note that a radially arranged series of fibrils can yield rhythmical changes in external curvature restricted to a single plane provided the pattern of excitation reaching the fibrils is bilaterally symmetrical about the desired plane of bending (see Appendix). If the fibrils pass straight down the tail, each fibril must be visualized as comparable to an earthworm, in that one region is relaxed when another region is contracted. The speed of conduction of a contractile wave would then be of the same order as the speed of conduction of the bending waves travelling along the tail, and the length of the bending wave determined by the length of fibril exhibiting one complete cycle of shortening. On the other hand, if the fibrils are spirally disposed along the length of the tail, the same mechanical result could be obtained from fibrils which shorten or lengthen simultaneously along their whole length; in this case the length of the bending wave would be determined by the length of tail covered by one complete turn of the spirally twisted fibrils.

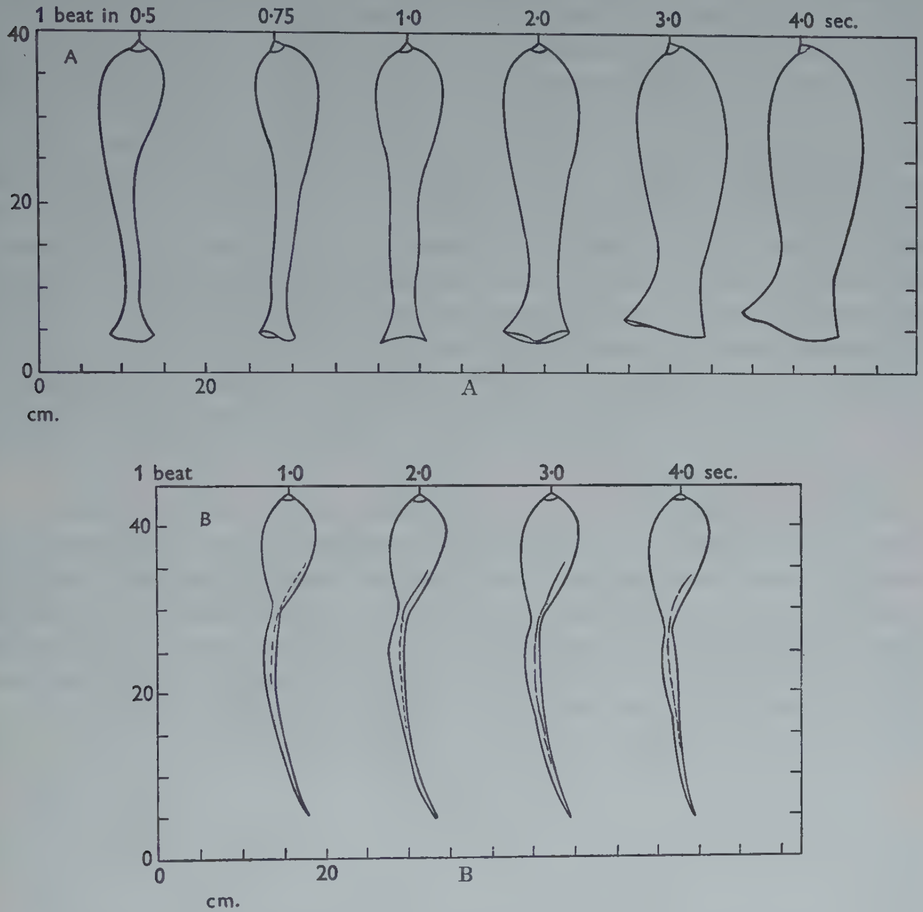
For many years two possibilities have been advocated concerning the mechanism whereby successive elements along a flagellum maintain a characteristic difference in phase of bending from that of their neighbours:

(i) That actively bending elements are restricted to the proximal region of the flagellum—the remainder of the flagellum representing an inert filament driven by the proximal active element, conduction along the flagellum depending on the latter's natural elasticity.

(ii) That all elements of the flagellum can actively change their shape and so contribute to the supply of energy necessary for propulsion.

The first hypothesis is based on the principle that bending waves can be passively propagated along an elastic filament immersed in water by oscillating one end by an external source of power; as already indicated, the form of a motionless sperm suggests that the tail possesses some degree of natural elasticity.

The similarity between the envelope of the waves passing down the tail with those passing passively along an elastic filament is illustrated by Text-fig. 13A. At first sight, therefore, the possibility of describing the movement of the sperm tail in terms of passive deformation of an elastic filament operated from a source of power localized at its proximal end seems worth consideration. There are, however, serious objections against this view. In the first place, the passive propagation of waves comparable in form to those exhibited by the active tail only occurs in an inert elastic filament if one end is constrained to move about a fixed hinge; in the spermatozoon the only source of restraint is the head. An actively bending element situated at the proximal end of the tail could not apply turning or bending forces to the rest of the tail greater than those which it applies to the head, and since the resistance of the water to movements of the head is probably relatively small compared with the resistance operating against the tail (see Gray & Hancock, 1955) an active element located close to the head could only oscillate the head from side to side without effecting any significant displacement of the rest of the tail. Two further differences exist between the movements of a passively oscillated filament and those of the sperm tail. In the case of the filament the form of the waves



Text-fig. 13. A. Envelope of waves propagated along a tapering strip of celluloid (37 cm. long, 0·025 cm. thick, and 2 cm. wide at proximal end). The strip was oscillated transversely in water through 90° at various frequencies about a fixed terminal hinge. Note the increase in width of the envelope with decreasing frequency of oscillation. Compare with Text-fig. 1 *a–h*. B. Envelope of waves propagated by the same strip in a more viscous medium. Note the damping effect on the distal end of the strip. Compare with Text-fig. 1 *i–l*.

(induced by a given type of terminal oscillation) depends on the frequency of the latter; the amplitude decreases markedly with increase of frequency; there is only slight evidence that this is so in the case of the sperm. Again, the form of the waves, and their ability to propagate themselves, is, in the case of an inert filament, markedly affected by the viscosity of the external medium (Fig. 13B). In a highly viscous medium, the envelope of the passive waves approximates in form to that of an 'ageing' spermatozoon—and the change is due to the damping effect of the medium. In the case of the sperm tail, envelopes of this type develop without any change in external viscosity. Finally, in drawing a comparison between the behaviour of a sperm tail and that of a passively oscillated rod, it is necessary to bear in mind that the behaviour of the latter is usually largely determined by its inertia. In the case of the sperm tail, the inertia of the system is negligible compared with the viscous resistance of the medium; the amount of kinetic energy present in an actively beating tail could not sustain its motion for more than a very small fraction of a second. All the evidence strongly suggests that the tail of a spermatozoon cannot be regarded as a passive rod oscillated by a localized source of power. The concept of sources of energy distributed along the whole tail—or at any rate the greater part of it—seems inescapable. At the same time, it is probably going too far to maintain that the passive elastic properties of the tail play no part in the mechanism of movement.

As the tail possesses natural elasticity, mechanical energy must be stored whenever an element is bent, and be released when it straightens out; it may well be that this energy is utilized in the useful work of propulsion and plays some part in the liberation of further energy for use by the contractile elements. It would be interesting to know whether the elastic energy stored in an element when it is bent is large or small in comparison with the total energy necessary to propel the element against the resistance of the medium. If a straight rod is bent into the arc of a circle the amount (U) of elastic energy stored is

$$U=\frac{EIl}{2r^2},$$

where E=Young's modulus, I='moment of inertia', l=length of rod, r=radius of curvature.

Applying this equation to a rod of the dimension of a sperm tail, each element of which bends into an arc of 4μ eighty times a second, and using a value of E of the same order as that determined by Mitchison & Swann (1954) for the cortex of sea-urchin eggs, suggests that the total rate of liberation of elastic energy by the tail is of the same order as that calculated by Rothschild (1953) for the propulsion of a mammalian spermatozoon at a comparable speed. The validity of this comparison is, of course, open to doubt, but the argument suggests that it may be unwise to assume that the passive storage of elastic energy by the tail is a minor consideration in the energetics of propulsion.

Reverting to the conclusion that a transformation of chemical into tensile energy occurs along the whole length of the tail, it is relevant to recall that regions of the tail mechanically isolated from the middle piece are invariably motionless. The middle piece thus appears to function as a 'kinetic centre' the activity of which is essential for the maintenance of the tail's movements. The instance quoted on p. 793 suggests, however, that the excitatory effect exerted by this centre on the tensile units of the tail cannot, in a sea-urchin spermatozoon, pass a region of mechanical restraint. It may be that the excitatory effect of the middle piece only leads to a liberation of tensile energy if the element concerned is mechanically stretched; under such conditions an active release of tensile energy would occur at the stage at which the amount of passively stored energy was at a maximum, and the rate of transmission of the wave would depend on the rate at which the total available energy could drive the element against the resistance of the water, and the form of the wave would depend at least in part on the natural rigidity of the tail. As an element moved from a position of maximum displacement towards the axis of propagation of the waves, the release of energy would be partly from an active source and partly from the energy already passively stored during the preceding process of bending; as the element moved away from the axis the energy available for propulsion would be the active energy minus the energy required for passive storage during the process of bending.

SUMMARY

1. The spermatozoa of *Psammechinus miliaris* (P. L. S. Müller; Gmelin) propel themselves by projecting transverse bending waves along their tails. All points on the tail normally execute their movements in approximately the same plane, their envelope forming a plane (or slightly twisted) lamina. The radius of maximum curvature is of the order of 4 μ.

2. In fresh suspensions at about 18° C. the waves are generated at a frequency of 30–40 per sec. and travel along the tail at a velocity of 800–1000 μ per sec. The average amplitude of the waves is 4 μ and the average wave-length 24 μ.

3. Elements of the tail situated near the head seldom bend to the same extent on their two sides. The symmetrical bending cycle of the central elements, on the other hand, sometimes imposes on the tail the form of a sine curve.

4. When moving over the surface of a glass slide the passage of each wave along the tail propels the head of the spermatozoon through a distance of 5–6 μ, and at the same time causes it to oscillate laterally through a distance of about 4 μ. The rate of forward propulsion of the head is seldom constant during all phases of the bending cycle; in extreme cases the head may move backwards during certain phases of the cycle. This asymmetry is probably due to the asymmetry of bending on the two sides of the tail.

5. Spermatozoa swimming over the surface of a slide travel at an average speed of about 190 μ per sec., but their path of progression is seldom straight; most cells travel along a curved track whose radius is usually 30–100 μ. Cells swimming farther away from the surface of a slide roll about their longitudinal axes with a frequency of 0·5–3·0 per sec., and their axis of progression is straight. The path of

a cell which yaws and rolls as it progresses forms a helix whose properties depend on the rate of yaw, frequency of roll and the rate of the cell's forward progression.

6. Eighty per cent of the cells moving over the surface of a slide yaw in a counter-clockwise direction relative to the observer. This phenomenon can be explained on the assumption that most of the cells when moving freely in a bulk of fluid behave as 'left-handed' screws rolling counter-clockwise as they advance; proximity to a surface prevents the cells from rolling and impresses on them an appearance of yawing in a counter-clockwise direction irrespective of whether they are yawing towards their right or left sides.

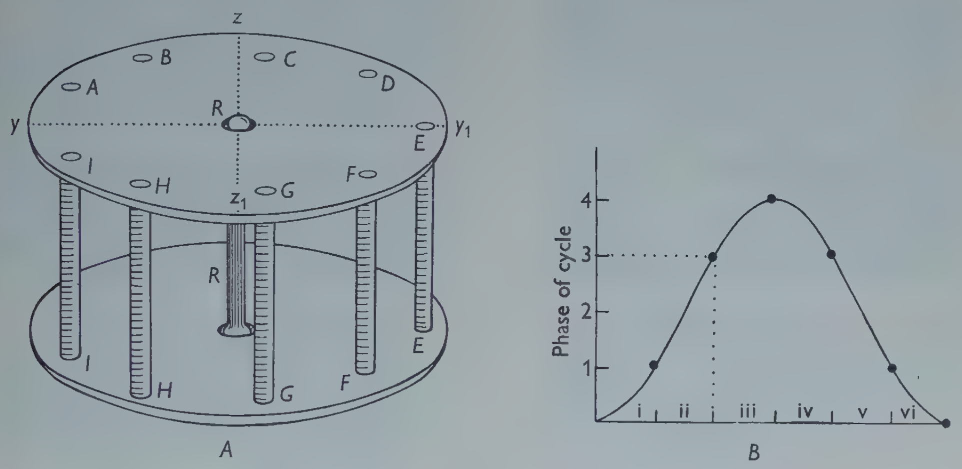
7. Evidence is presented which supports the view that all regions of the tail are actively contractile although mechanical forces may affect the propagation of bending waves along the filament.

My very sincere thanks are due to a number of colleagues, particularly to Prof. M. M. Swann for his help with some of the earlier photographs; to Prof. T. Fox and Dr K. E. Machin for help with the physical aspects of the problem; and to Lord Rothschild for many fruitful discussions. Finally, I owe the whole of the cinematograph recording to the patience and skill of Mr K. Williamson.

APPENDIX

Text-fig. 14*A* illustrates a mechanical system analogous to a short segment of the tail of a spermatozoon. Two circular plates are hinged concentrically by universal joints to the ends of a rigid rod (*R*), the plates and the rod representing the compression elements of the tail. The plates are also connected by nine radially arranged contractile fibres (*A*–*I*). If the top plate is to oscillate about one axis (ZZ_1) only, the distribution of tension between the individual fibres must conform with two conditions; (i) the resultant of all the tensile forces exerted by the fibres shall (together with force exerted by the rod *R*) yield a couple, about the axis of oscillation, which varies rhythmically in magnitude and direction during each cycle of oscillation; (ii) at no phase in the oscillation shall there be any resultant couple about an axis (yy_1) normal to the axis of oscillation (zz_1). The first of these conditions is satisfied if each fibre undergoes a contractile cycle identical with that of all the others but maintains a constant phase difference from its immediate neighbours. The second condition is satisfied if the distribution of tension between the different fibres is such that it is bilaterally symmetrical about an axis (yy_1) normal to the axis of oscillation (zz_1). These principles are illustrated by the following example.

Let the form of the contractile cycle of each element approximate to that of a sine wave and produce a maximum tension of four arbitrary units and let the phase difference between adjacent fibres be one-sixth of a cycle. Each cycle can then be divided into six equal intervals of time and the relative levels of tension at the beginning of each of the six consecutive intervals will be approximately 0, 1, 3, 4, 3, 1 (Text-fig. 14*b*). If the conditions prescribed above are to be fulfilled, the distribution of tension throughout the whole contractile cycle must be that shown in Table 4, which shows that each fibre is always one-sixth of a cycle out of phase with its immediate neighbour and that the distribution of tension is bilaterally symmetrical about the radial axis (*yy*) passing through fibre *E*; fibre *A* is always in phase with fibre *I*, fibre *B* with fibre *H*, fibre *C* with fibre *G*, and fibre *D* with fibre *F*.



Text-fig. 14.

Table 4

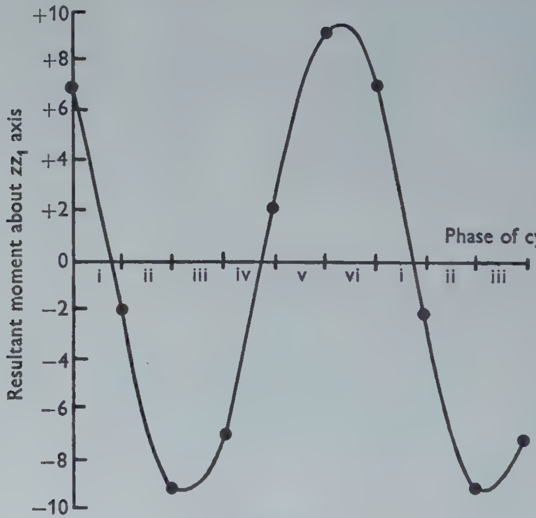
Fibril	Tension at beginning of successive phases					
	i	ii	iii	iv	v	vi
A	3	1	0	1	3	4
B	4	3	1	0	1	3
C	3	4	3	1	0	1
D	1	3	4	3	1	0
E	0	1	3	4	3	1
F	1	3	4	3	1	0
G	3	4	3	1	0	1
H	4	3	1	0	1	3
I	3	1	0	1	3	4

For the sake of convenience the cycle is assumed to start when the tension of fibre *E* is zero.

That this distribution of tension maintains a regular oscillation about zz_1 is shown by taking moments of all the tensions about zz_1; tensions exerted by fibres *A*, *B*, *H*, *I* are denoted as 'positive', and those by *C*, *D*, *E*, *F* and *G* as 'negative'. These moments together with their resultant value at the beginning of each phase of the cycle are shown in Table 5 and Text-fig. 15; one oscillation occurs during each cycle of fibrillar contraction.

Table 5

Fibril	Sign of moment	Moments at beginning of phase					
		i	ii	iii	iv	v	vi
A	Positive	2·82	0·94	0	0·94	2·82	3·76
B	Positive	2·00	1·50	0·50	0	0·50	1·50
C	Negative	0·522	0·696	0·522	0·174	0	0·174
D	Negative	0·766	2·298	3·064	2·298	0·766	0
E	Negative	0	1·000	3·000	4·000	3·000	1·000
F	Negative	0·766	2·298	3·064	2·298	0·766	0
G	Negative	0·522	0·696	0·522	0·174	0	0·174
H	Positive	2·00	1·50	0·50	0	0·50	1·50
I	Positive	2·82	0·94	0	0·94	2·82	3·76
Total		+7·064	−2·108	−9·172	−7·064	+2·108	+9·172



Text-fig. 15.

REFERENCES

Afzelius, B. A. (1955). The fine structure of the sea-urchin spermatozoon as revealed by the electron-microscope. *Z. Zellforsch.* **42**, 134–48.

Bradfield, J. R. G. (1953). New features of protoplasmic structure observed in recent electron microscope studies. *Quart. J. Micr. Sci.* **94**, 351–67.

Buller, A. H. R. (1903). Is chemotaxis a factor in the fertilization of the eggs of animals? *Quart. J. Micr. Sci.* **46**, N.S., 154–76.

Dewitz, J. (1886). Über Gesetzmässigkeit in der Ortsveränderung der Spermatozoen und in der Versinigung mit dem Ei. *Arch. ges. Physiol.* **38**, 358–85.

Gray, J. (1953). Undulatory propulsion. *Quart. J. Micr. Sci.* **94**, N.S., 551–78.

Gray, J. & Hancock, G. J. (1955). The propulsion of sea-urchin spermatozoa. *J. Exp. Biol.* **32**, 802–14.

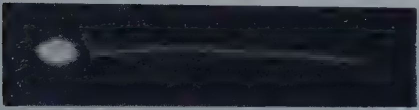
Mitchison, J. M. & Swann, M. M. (1954). The mechanical properties of the cell-surface. II. *J. Exp. Biol.* **31**, 461–72.

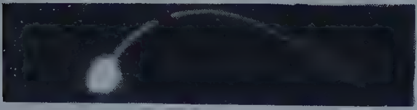
Rothschild, Lord (1951). Sea urchin spermatozoa. *Biol. Rev.* **26**, 1–27.

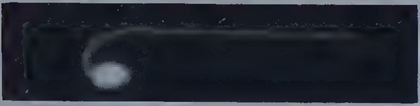
Rothschild, Lord (1953). *Mammalian Germ Cells*, pp. 122–30. London: Churchill.

Rothschild, Lord & Swann, M. M. (1949). The fertilization reaction in the sea-urchin egg. *J. Exp. Biol.* **26**, 164–76.

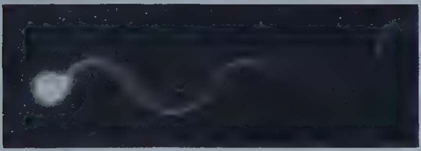
Uhlela, V. (1911). Ultramikroskopische Studien über Geisselbewegung. *Biol. Zbl.* **31**, 645–76.

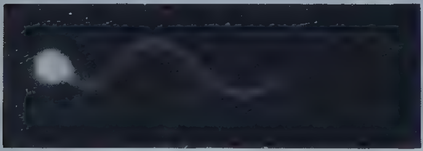
1


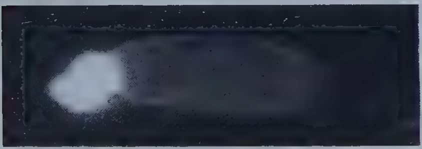
2


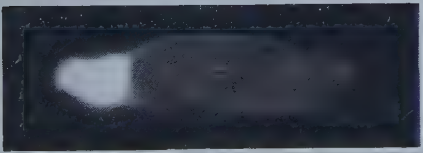
3


4

5


6


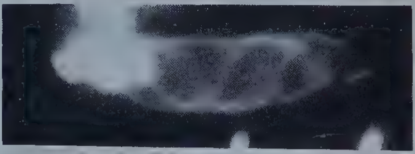
7


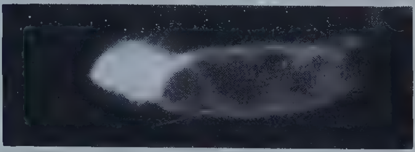
8


9

10

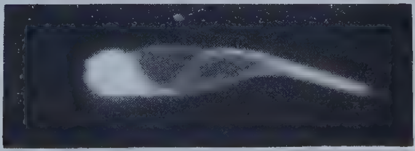
11

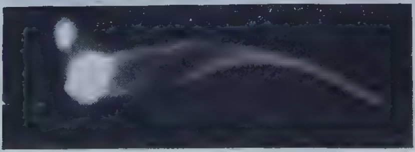
12


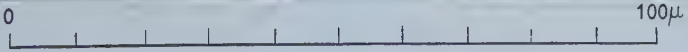
13


14

15

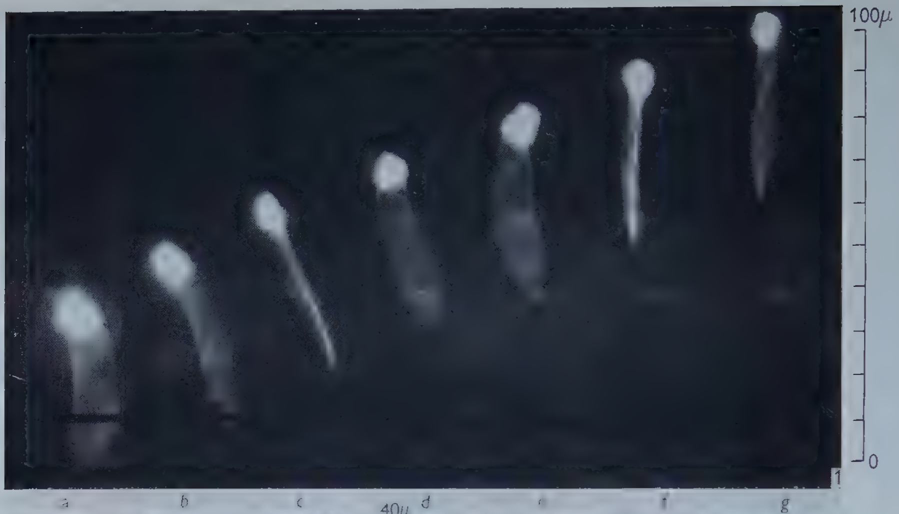
16


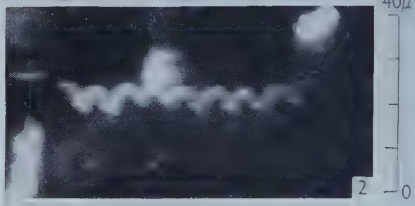
17


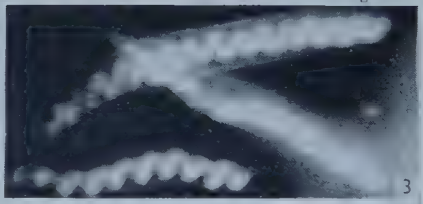


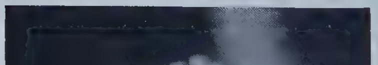
RAY—THE MOVEMENT OF SEA-URCHIN SPERMATOZOA

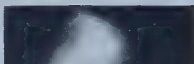
(*Facing p.* 800)

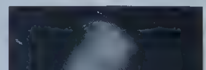












GRAY—THE MOVEMENT OF SEA-URCHIN SPERMATOZOA

EXPLANATION OF PLATES

PLATE 3

Figs. 1–3. Dead or motionless spermatozoa. Figs. 4–13. Active spermatozoa from fresh suspensions. Figs. 14–17. From older suspensions.

The approximate exposure of each photograph is shown in fractions of a second.

Figs. 7, 8, 9, 14, 15 are photographs taken with an arc of steady voltage. Figs. 10–13; 16, 17 were taken with a variable arc.

PLATE 4

Fig. 1. Seven successive photographs (*a–g*) from a cinematograph record (12 frames per sec.) of a spermatozoon rolling about its longitudinal axis 2·0 times per sec.

Figs. 2–6. Tracks of heads.

Figs. 3, 4. 5. Showing envelopes of tails.

THE PROPULSION OF SEA-URCHIN SPERMATOZOA

By J. GRAY* and G. J. HANCOCK†

(*Received* 20 *May* 1955)

The movement of any short length of the tail of a spermatozoon of *Psammechinus miliaris* and the characteristic changes which it undergoes during each cycle of its displacement through the water can be described in terms of the form and speed of propagation of the bending waves which travel along the tail (Gray, 1953, 1955); the form of the wave depends on the maximum extent of bending reached by each element and on the difference in phase between adjacent elements. The object of this paper is to consider the forces exerted on the tail as it moves relative to the surrounding medium and to relate the propulsive speed of the whole spermatozoon to the form and speed of propagation of the bending waves generated by the tail. The mathematical theory of the propulsive properties of thin undulating filaments has recently been considered by Taylor (1951, 1952) and by Hancock (1953); the present study utilizes and extends their findings but approaches the problem from a somewhat different angle.

I. GENERAL THEORY

As in all self-propelling undulatory systems, the propulsion of a spermatozoon depends on the fact that the retarding effect of all the tangential forces acting along the body is compensated by propulsive components of forces acting normally to the surface of the body (Gray, 1953). Any region of the body eliciting from the water a reaction normal to its surface must have a component of motion normal to this surface, and—if this reaction is to have a forward propulsive component along the axis of translation of the whole cell—the region or element concerned must have an appropriate orientation to this axis (Gray, 1953). To apply this principle to the tail of a spermatozoon it is convenient to consider the forces exerted on a short element (δs) by virtue of the transverse displacement (V_y) impressed on it during the passage of a wave. As explained elsewhere (Gray, 1953), the orientation (θ) of an element to the axis of propulsion (xx_1) depends on the form of the wave and on the element's position on the wave; the element's transverse velocity (V_y) depends on the form of the waves and on their speed of propagation (V_w). An element of this type is shown in Fig. 1A; its velocity along the axis yy' is V_y and its surface is inclined to the axis (xx') of propulsion by an angle θ. The transverse displacement (V_y) has two components: (i) a tangential displacement ($V_y \sin\theta$), and (ii) a displacement ($V_y \cos\theta$) normal to the surface of the element; to both these displacements the water offers resistance, and consequently the transverse displacement (V_y) elicits reactions tangential and normal to the surface of the element. The latter force (δN_y) has a component ($\delta N_y \sin\theta$) acting forward along the axis (xx') of propulsion; it is this component which counteracts the retarding effect of all the forces acting tangentially to the surface.

---

* Department of Zoology, University of Cambridge.

† Department of Aeronautical Engineering, Queen Mary College, University of London.

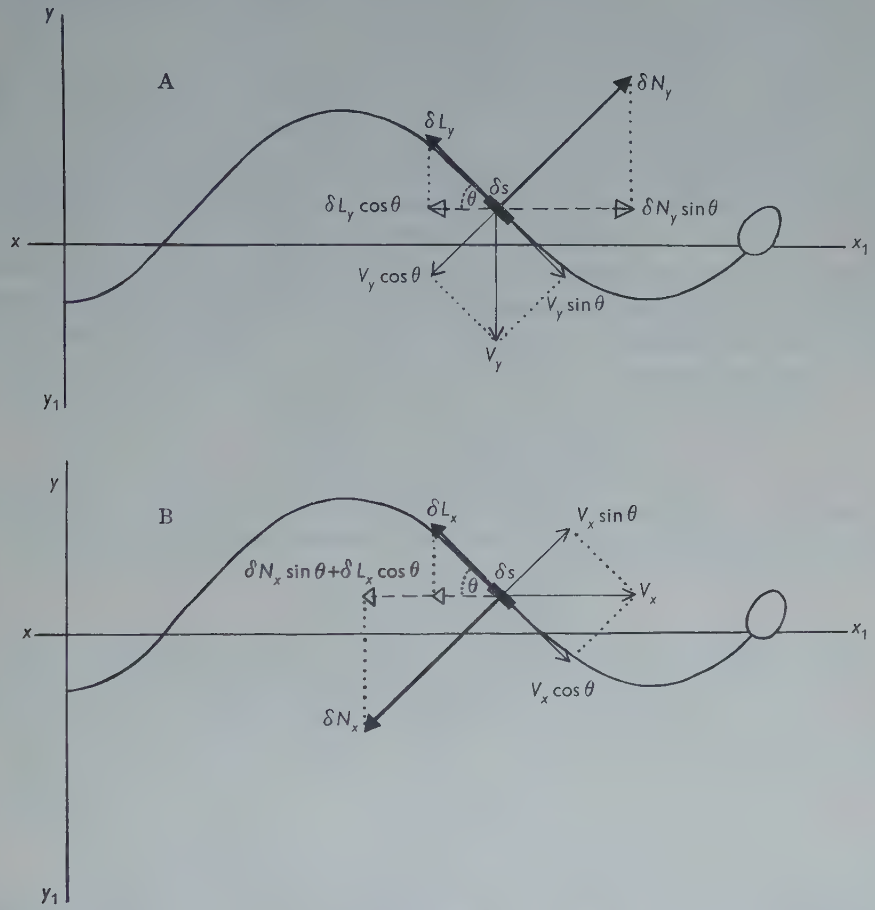


Fig. 1. A. Diagram illustrating the forces impressed on an element δs when moving transversely across the axis of progression (xx_1) at velocity V_y, the resultant propulsive thrust (δF_y) being $\delta N_y \sin\theta - \delta L_y \cos\theta$, where δN_y and δL_y are the reactions from the water acting normally and tangentially to the surface of the element, and θ is the angle of inclination of the element to the axis xx_1. For values of N_y and L_y see text. B. Diagram illustrating the forces impressed on an element when displaced along the xx_1 axis at velocity V_x, the resultant drag (δF_x) being $\delta N_x \sin\theta + \delta L_z \cos\theta$. The net propulsive thrust (δF) due to simultaneous transverse and forward movement is obtained by combining A and B.

As the dimensions of an element are extremely small and the speed of displacement very low, the reactions elicited from the water can (by analogy with those operating on a sphere) be regarded as directly proportional to the velocity of displacement and to the viscosity of the medium. If the velocity of displacement tangential to the body is $V_y \sin\theta$, the tangential drag (δL_y) can be expressed as $C_L V_y \sin\theta\delta s$, and the force ($\delta N_y$) acting normally to the surface as $C_N V_y \cos\theta\delta s$, where C_L and C_N are the coefficients of resistance to the surface of the element for a medium of known viscosity; the resultant forward thrust (δF_y) along the axis (xx') of propulsion is $(C_N - C_L) V_y \sin\theta \cos\theta\delta s$. In other words, the transverse displacement of the element will produce a forward thrust along the axis of propulsion provided the coefficient of resistance to displacement, normal to the surface of the element, is greater than that to displacement along the surface.

In an actively moving spermatozoon, each element is not only moving transversely across the axis of propulsion, it is also moving along the latter axis at a speed (V_x) which depends upon the speed at which the whole spermatozoon is progressing through the water (Fig. 1 B). This forward displacement being equivalent to a tangential displacement $V_x \cos\theta$ and a normal displacement $V_x \sin\theta$, the corresponding forces acting tangentially (δL_x) and normally (δN_x) to the surface are $C_L V_x \cos\theta\delta s$ and $C_N V_x \sin\theta\delta s$ respectively. The total forces (δN and δL), acting normally and tangentially to the surface owing to the element's transverse and forward displacements, are

$$\left.\begin{aligned} \delta N &= C_N(V_y \cos\theta - V_x \sin\theta)\,\delta s, \\ \delta L &= C_L(V_y \sin\theta + V_x \cos\theta)\,\delta s. \end{aligned}\right\} \tag{i}$$

The propulsive components of δN and δL along the axis of propulsion (xx') being $\delta N \sin\theta$ and $\delta L \cos\theta$ respectively, the resultant forward thrust (δF) is

$$\delta N \sin\theta - \delta L \cos\theta:$$

$$\begin{aligned} \delta F &= [(C_N - C_L) V_y \sin\theta \cos\theta - V_x(C_N \sin^2\theta + C_L \cos^2\theta)]\,\delta s \\ &= \left[\frac{(C_N - C_L) V_y \tan\theta - V_x(C_L + C_N \tan^2\theta)}{1 + \tan^2\theta}\right]\delta s. \end{aligned} \tag{ii}$$

Equation (i) shows the element can only exert a positive forward thrust if

$$V_y > V_x \tan\theta.^*$$

Equation (ii) shows that a positive forward thrust only develops if $C_N > C_L$.

During steady motion the resultant thrust (δF) from an element, when integrated over a complete cycle, is zero; it is now possible to express the propulsive speed in terms of the wave speed, if the values of C_N, C_L and θ are known and if the propulsive speed remains constant during the whole cycle of the element's motion.

---

* If $V_y = V_x \tan\theta$ the element has no component of motion normal to its surface; motion of this type could only ensue if the element were exerting its effort against a rigid medium—and were equivalent to a snake gliding through a rigid close-fitting tube.

II. PROPULSIVE EFFECT OF WAVES OF SMALL AMPLITUDE

The possibility of applying the above principles to an undulating flagellum was first pointed out to one of us (J. G.) in 1933 by the late Dr R. C. Howland. If the transverse velocity of an element be dy/dt and the tangent of its angle of inclination be dy/dx, equation (ii) becomes

$$dF=\left\{\frac{(C_N-C_L)\dfrac{dy}{dt}\dfrac{dy}{dx}-V_x\left[C_L+C_N\left(\dfrac{dy}{dx}\right)^2\right]}{1+\left(\dfrac{dy}{dx}\right)^2}\right\}ds, \tag{iii}$$

the total thrust (F) exerted by the flagellum over one wave-length $0\leqslant x\leqslant\lambda$ is

$$F=\int_0^\lambda dF. \tag{iv}$$

To proceed further Howland restricted attention to cases in which the angle of inclination of an element was sufficiently small to eliminate terms containing $(dy/dx)^2$ and to regard the length of the flagellum constituting one wave-length as approximately equal to the wave-length. Equation (iii) then reduces to

$$\frac{dF}{dx}=(C_N-C_L)\frac{dy}{dt}\frac{dy}{dx}-C_L V_x. \tag{v}$$

If the form of the waves generated by the tail conforms to that of a sine curve

$$y=b\sin\frac{2\pi}{\lambda}(x+V_w t),$$

where b = amplitude, λ = wave-length, V_w = velocity of wave relatively to the head. The transverse velocity (dy/dt) of the element is $\dfrac{2\pi b V_w}{\lambda}\cos\dfrac{2\pi}{\lambda}(x+V_w t)$ and the tangent of the angle of inclination (dy/dx) of the element is $\dfrac{2\pi b}{\lambda}\cos\dfrac{2\pi}{\lambda}(x+V_w t)$

$$\frac{dF}{ds}\approx\frac{dF}{dx}=(C_N-C_L)\frac{4\pi^2b^2V_w}{\lambda^2}\cos^2\frac{2\pi}{\lambda}(x+V_w t)-C_L V_x,$$

$$F=\int_0^\lambda dF=\frac{2\pi^2b^2V_w(C_N-C_L)}{\lambda}-C_L\lambda V_x. \tag{vi}$$

Equation (vi) gives the forward thrust exerted by each complete wave.

When an undulating organism (without a head) is propelling itself forward at a steady speed (V_x), $F=0$

$$\frac{V_x}{V_w}=\frac{2\pi^2b^2}{\lambda^2}\left(\frac{C_N-C_L}{C_L}\right), \tag{vii}$$

or since $V_w=f\lambda$ (where f is the frequency of the waves)

$$V_x=\frac{2f\pi^2b^2}{\lambda}\left(\frac{C_N-C_L}{C_L}\right). \tag{viii}$$

The active tail of a spermatozoon exerts its effort against the drag of an inert head, and consequently

$$nF=D, \tag{ix}$$

where n is the number of waves exhibited simultaneously by the tail and D is the drag of the head. The drag of the head can be denoted by $hC_L aV_x$, where hC_L is the drag coefficient and a is a linear dimension of the head. From equations (vi) and (ix)

$$\frac{2n\pi^2 b^2 V_w}{\lambda}(C_N-C_L)-C_L V_x(n\lambda+ha)=0,$$

$$\frac{V_x}{V_w}=\frac{2\pi^2 b^2}{\lambda^2}\left(\frac{C_N-C_L}{C_L}\right)\left(\frac{1}{1+\dfrac{ha}{n\lambda}}\right), \tag{x}$$

or

$$V_x=\frac{2f\pi^2 b^2}{\lambda}\left(\frac{C_N-C_L}{C_L}\right)\left(\frac{1}{1+\dfrac{ha}{n\lambda}}\right). \tag{xi}$$

Howland thus reached the conclusion that an organism comparable with a spermatozoon should propel itself through the water at a speed which depended on six factors: (1) the frequency of the waves; (2) the square of the amplitude of the waves and (3) their wave-length; (4) the difference between the coefficients of normal and tangential resistance; (5) the drag coefficient and size of the head; (6) the length of the tail.

Until recently, Howland's results appeared to have only limited significance from an experimental point of view, for, apart from the limitation to waves of small amplitude, there seemed no means of determining the values of C_N, C_L and h and no observational data on the amplitude, wave-length and frequency of the waves. It is now known, however (Hancock, 1953), that for very thin filaments (such as the tail of a spermatozoon) C_N is effectively twice C_L. It is also known that the waves passing down the tail of a spermatozoon of *Psammechinus* sometimes conform closely to sine waves of amplitude 4μ and wave-length 24μ, and that their frequency is about 30–40 per sec. (Gray, 1955).

It will be noted that if $C_N=2C_L$ equation (vii) becomes

$$\frac{V_x}{V_w}=\frac{2\pi b^2}{\lambda^2}. \tag{xii}$$

Equation (vii) is thus identical with that derived by Taylor (1952) and by Hancock (1953) for waves of small amplitude. Howland's argument has been set out in some detail in view of the fact that its underlying physical principles can readily be visualized from a biological point of view.

III. PROPULSIVE THRUST FROM WAVES OF RELATIVELY LARGE AMPLITUDE

If a filament is deformed into a single complete sine curve of relatively large amplitude, the length (s) of the filament is considerably greater than the wave-length (λ), and the length (δs) of the filament intercepted by a short distance (δx) along the axis of propulsion varies with the phase of transverse motion. Consequently each point on a non-extensible filament executes a figure of eight movement relative to the head of the filament. The motion of each element relative to fixed axes is therefore a figure of eight superimposed upon a forward propulsive velocity ($\overline{V}_x$) defined by the velocity of forward propulsion when averaged over a complete cycle of activity.

The length of filament (δs) intercepted by a small fraction (δx) of a wave-length is given by equation (xiii)

$$(\delta s)^2 = (\delta x)^2 + (\delta y)^2,$$

$$\delta s = \left[1 + \left(\frac{dy}{dx}\right)^2\right]^{\frac{1}{2}} \delta x. \tag{xiii}$$

Equation (iii) now becomes equation (xiv)

$$dF = \frac{\left\{(C_N - C_L)\dfrac{dy}{dt}\dfrac{dy}{dx} - \overline{V}_x\left[C_L + C_N\left(\dfrac{dy}{dx}\right)^2\right]\right\}}{\left[1 + \left(\dfrac{dy}{dx}\right)^2\right]^{\frac{1}{2}}} dx. \tag{xiv}$$

If $C_N = 2C_L$

$$dF = \frac{C_L\left\{\dfrac{dy}{dt}\dfrac{dy}{dx} - \overline{V}_x\left[1 + 2\left(\dfrac{dy}{dx}\right)^2\right]\right\}}{\left[1 + \left(\dfrac{dy}{dt}\right)^2\right]^{\frac{1}{2}}} dx \tag{xv}$$

For a sine wave

$$\frac{dy}{dx} = \frac{2\pi b}{\lambda}\cos\frac{2\pi}{\lambda}(x + V_w t),$$

$$\frac{dy}{dt} = \frac{2\pi b V_w}{\lambda}\cos\frac{2\pi}{\lambda}(x + V_w t).$$

Substituting in equation (xv) and denoting dy/dx as A

$$dF = \frac{C_L[V_w A^2 - \overline{V}_x(1 + 2A^2)]}{(1 + A^2)^{\frac{1}{2}}} dx. \tag{xvi}$$

The forward thrust (F) from one complete wave is $\int_{x=0}^{x=\lambda} dF$,

$$F = C_L\left[V_w \int_0^\lambda \frac{A^2}{(1 + A^2)^{\frac{1}{2}}} dx - \overline{V}_x \int_0^\lambda \frac{1 + 2A^2}{(1 + A^2)^{\frac{1}{2}}} dx\right]. \tag{xvii}$$

Putting $\int_0^\lambda \frac{A^2}{(1 + A^2)^{\frac{1}{2}}} dx = I$ and $\int_0^\lambda \frac{1 + 2A^2}{(1 + A^2)^{\frac{1}{2}}} dx = J$,

$$F = V_w I - \overline{V}_x J. \tag{xviii}$$

If $F=0$
$$\frac{\overline{V}_x}{V_w}=\frac{I}{J}, \tag{xix}$$

where
$$I=\int_0^\lambda\left\{\frac{\dfrac{4\pi^2b^2}{\lambda^2}\cos^2\dfrac{2\pi}{\lambda}(x+V_wt)}{\left[1+\dfrac{4\pi^2b^2}{\lambda^2}\cos^2\dfrac{2\pi}{\lambda}(x+V_wt)\right]^{\frac{1}{2}}}\right\}dx$$

and
$$J=\int_0^\lambda\left\{\frac{1+\dfrac{8\pi^2b^2}{\lambda^2}\cos^2\dfrac{2\pi}{\lambda}(x+V_wt)}{\left[1+\dfrac{4\pi^2b^2}{\lambda^2}\cos^2\dfrac{2\pi}{\lambda}(x+V_wt)\right]^{\frac{1}{2}}}\right\}dx.$$

If $\cos^2\frac{2\pi}{\lambda}(x+V_wt)$ is replaced by the equivalent expression $\frac{1}{2}\left[1+\cos\frac{4\pi}{\lambda}(x+V_wt)\right]$, the contribution to the integrals I and J of the term $\cos\frac{4\pi}{\lambda}(x+V_wt)$ will be smaller than the remaining terms; if this term is neglected I/J approximates (over all the range of values of $2\pi b/\lambda$) to

$$\frac{2\pi^2b^2}{\lambda^2}\left(\frac{1}{1+\dfrac{4\pi^2b^2}{\lambda^2}}\right);$$

hence
$$\frac{\overline{V}_x}{V_w}=\frac{2\pi^2b^2}{\lambda^2}\left(\frac{1}{1+\dfrac{4\pi^2b^2}{\lambda^2}}\right), \tag{xx}$$

or
$$\overline{V}_x=\frac{2f\pi^2b^2}{\lambda}\left(\frac{1}{1+\dfrac{4\pi^2b^2}{\lambda^2}}\right). \tag{xxi}$$

It will be noted that equation (xx) is identical with that already obtained from a different line of approach (Hancock, 1952, p. 106) and yields the same result as equation (xii) for waves of small amplitude.

IV. UNDULATING FILAMENT PROPELLING AN INERT HEAD

In the case of a filament propelling an inert head

$$n\int_0^\lambda dF-C_H\overline{V}_x=0, \tag{xxii}$$

where n = number of waves exhibited by the whole tail and C_H = drag coefficient of the head. Assuming the head to be spherical $C_H=6\pi a\mu$, where a is the radius of the head and μ the viscosity of the medium.

From equations (xvii) and (xxii)

$$nV_wC_L\int_0^\lambda\frac{A^2}{(1+A^2)^{\frac{1}{2}}}dx=\overline{V}_x\left[nC_L\int_0^\lambda\frac{1+\dfrac{C_N}{C_L}A^2}{(1+A^2)^{\frac{1}{2}}}dx+6\pi a\mu\right].$$

If $C_N = 2C_L$

$$nV_w C_L \int_0^\lambda \frac{A^2}{(1+A^2)^{\frac{1}{2}}}\,dx = \overline{V}_x\left[nC_L\int_0^\lambda \frac{1+2A^2}{(1+A^2)^{\frac{1}{2}}}\,dx + 6\pi a\mu\right], \tag{xxiii}$$

where

$$A = \frac{2\pi b}{\lambda}\cos\frac{2\pi}{\lambda}(x+V_w t).$$

To utilize equation (xxiii) it is necessary to know the absolute value of C_L. This can be derived from equation (7) of a previous paper (Hancock, 1953, p. 102), which gives the fluid velocity V in the vicinity of an element of length δs moving with a velocity V_t tangentially to itself in a fluid which is at rest:

$$V = V_t\frac{\left(\log\dfrac{r}{2\lambda}\right)+\frac{1}{2}}{\left(\log\dfrac{d}{2\lambda}\right)+\frac{1}{2}},$$

where V is the velocity of the fluid parallel to the element surface, d = the radius of the filament, r = the distance from the filament surface, $2\pi/k = \lambda$ = the wave-length.

The tangential drag force dF on the element is therefore

$$dF = \frac{2\pi\mu V_t . ds}{\left(\log\dfrac{d}{2\lambda}\right)+\frac{1}{2}},$$

$$C_L = -\frac{2\pi\mu}{\left(\log\dfrac{d}{2\lambda}\right)+\frac{1}{2}}. \tag{xxiv}$$

Substituting this value for C_L in (xxiii) and using the integrals already defined

$$\frac{\overline{V}_x}{V_w} = \frac{I}{J - \dfrac{3a}{n\lambda}\left[\left(\log\dfrac{d}{2\lambda}\right)+\frac{1}{2}\right]},$$

reducing approximately, by a similar argument as before, to

$$\frac{\overline{V}_x}{V_w} = \frac{2\pi^2 b^2}{\lambda^2}\left\{\frac{1}{1+\dfrac{4\pi^2 b^2}{\lambda^2} - \left(1+\dfrac{2\pi^2 b^2}{\lambda^2}\right)^{\frac{1}{2}}\dfrac{3a}{n\lambda}\left[\left(\log\dfrac{d}{2\lambda}\right)+\frac{1}{2}\right]}\right\}, \tag{xxv}$$

or

$$\overline{V}_x = \frac{2f\pi^2 b^2}{\lambda}\left\{\frac{1}{1+\dfrac{4\pi^2 b^2}{\lambda^2} - \left(1+\dfrac{2\pi^2 b^2}{\lambda^2}\right)^{\frac{1}{2}}\dfrac{3a}{n\lambda}\left[\left(\log\dfrac{d}{2\lambda}\right)+1\right]}\right\}. \tag{xxvi}$$

V. APPLICATION OF THEORETICAL ANALYSIS TO OBSERVATIONAL DATA

The calculated propulsive speed of the spermatozoa of *P. miliaris* can be derived from equation (xxvi) by utilizing the data provided in a previous paper (Gray, 1955), bearing in mind the uncertainty of some of the values involved.

As a general test of agreement between the calculated and observed speeds, the following values will be used:

Amplitude of waves	(b)	$4\,\mu$
Length of waves	(λ)	$24\,\mu$
No. of simultaneous waves	(n)	1·3
Frequency of waves	(f)	35 per sec.
Radius of head	(a)	$0\cdot5\,\mu$
Radius of tail	(d)	$0\cdot2\,\mu$

From equation (xxvi)
$$\overline{V}_x = 462\,\frac{1}{1+1\cdot1+0\cdot3}$$
$$=\frac{462}{2\cdot4}=191\,\mu \text{ per sec.}$$

The average observed speed (as derived from 89 cells) was 191·4 μ per sec. for an average frequency of 34·5 per sec. For thirty-three of these cells the speed of propulsion was determined photographically, whilst the frequency was measured stroboscopically; for 56 cells both speed and frequency were measured photographically. The significance of the very remarkable agreement between the calculated and observed results must not be over-emphasized. Quite apart from the theoretical considerations referred to below, it must be borne in mind that the values used for the effective radius of the head and tail are necessarily approximate, and that most of the other values represent the arithmetic mean of populations showing considerable variation between individuals; thus in a population of 29 cells the frequency of the waves varies between 25 and 46 per sec., and in the population of 89 cells referred to above, the propulsive speed varied from 100 to 290 μ per sec. Probably the safest estimate of agreement between observed and calculated values is provided by the ratio $\overline{V}_x/V_w$, since this eliminates any error in an estimate of wave frequency; the calculated value of 0·23 indicates that the passage of each wave of $24\,\mu$ wave-length over the tail should propel the spermatozoon through a distance of $5\cdot52\,\mu$; a very large number of records show that the observed distance lies between 5 and $6\,\mu$.

Table 1

No. of cells	Average freq. per sec (f)	Speed of wave (μ per sec) $(V_w=24f)$	Speed of propulsion (μ per sec.)		Ratio $(\overline{V}_x/V_w)$	
			Calc.	Obs.	Calc.	Obs.
33	36·9	886	201	208	0·23	0·20–0·25
56	33	792	180	181	0·23	0·20–0·25

VI. DISCUSSION

The theoretical analysis involves at least three assumptions which differ from the observed facts:

(i) The form of the wave—both in amplitude and wave-length—only conforms to a sine curve during part of its propagation along the tail; the movements executed by elements of the tail at the two ends are different from those situated more centrally. The values given for amplitude and wave-length are, therefore, average values not only for a number of cells but also averages over one complete cycle. As shown in the Appendix, however, this limitation is not likely to be serious as far as variations in amplitude are concerned.

(ii) In order that the form of the tail should conform to that of a sine wave the changes in curvature and rate of transverse displacement about the axis of progression must be symmetrical. In many instances this is not the case (Gray, 1955).

(iii) The formula assumes that the head travels along the axis of progression without oscillating from side to side. In fact, the head always oscillates during each cycle for a distance comparable with its forward displacement. It may be noted, however, that on theoretical grounds the effect of the head on the rate of forward propulsion is likely to be small. Equation (xxvi) indicates that the presence of the head of the sperm of *P. miliaris* only reduces the speed of propulsion by about 15 %. In the absence of the head, the last term in the denominator becomes zero, and the calculated speed of progression at 35 waves per sec. rises to about 220 μ per sec. In other words, the propulsive component of the forces acting normally to the surface of the tail largely operates against the tangential drag of the tail—and only to a minor extent against the drag of the head.

Finally, it must be remembered that all the data on speeds of translation are derived from spermatozoa which are moving in close proximity to a glass or air surface, and the assumption is made that these figures also apply to spermatozoa moving freely in a bulk of fluid.

Bearing in mind the above limitations it seems, nevertheless, remarkable that the forward speed of propulsion of a spermatozoon can be calculated (well within the limits of experimental error) in terms of the form and frequency of the bending waves generated by the tail, on the basis that the coefficient of resistance of displacement of the tail normal to its surface is twice that of its displacement tangential to this surface and that all regions of the tail contribute equally towards the necessary propulsive thrust.

APPENDIX

Effect of variation in amplitude of waves

In many undulatory organisms the amplitude of the waves tends to increase as the waves pass posteriorly along the body (Gray, 1953). This feature can be incorporated into the theoretical analysis by assuming that the wave form is described by

$$y=b(x)\sin k(x+V_w t),$$

where the amplitude function $b(x)$ depends on x, and $k=2\pi/\lambda$.

The increment of force dF acting in the x direction may be defined by a similar argument as before:

$$\frac{dF}{dx}=\frac{V_w(C_N-C_L)\,[b^2k^2\cos^2 k(x+V_w t)+\tfrac{1}{2}bb'k\sin 2k(x+V_w t)]}{\{1+[bk\cos k(x+V_w t)+b'\sin k(x+V_w t)]^2\}^{\frac{1}{2}}}$$
$$-\frac{\overline{V}_x\{C_N[bk\cos k(x+V_w t)+b'\sin k(x+V_w t)]^2+C_L\}}{\{1+[bk\cos k(x+V_w t)+b'\sin k(x+V_w t)]^2\}^{\frac{1}{2}}}, \qquad (1\,a)$$

where the dash denotes differentiation with respect to x. The velocity of propulsion $\overline{V}_x$ can be obtained from equation (1 *a*) by taking the integral $\int dF=0$ as before. A number of applications are now developed:

(i) Consider a semi-infinite filament ($0\leqslant x\leqslant\infty$) with steadily decreasing amplitude as x increases, assuming that

$$b(x)=b\,e^{-kx/a},$$

then

$$b^1=\frac{-bk}{a}e^{-kx/a},$$

where a is the parameter defining the rate at which the amplitude changes and in particular the ratio of any two amplitudes distance one wave-length apart is $e^{-2\pi\,a}$; $a=\infty$ gives the case of constant wave-length. This wave form is shown below in Fig. 2. Hence from equation (1 *a*), using the fact that for extremely thin filaments $C_N=2C_L$,

$$\frac{\overline{V}_x}{V_w}=\frac{I}{J},$$

where

$$I=\int_0^\infty\frac{\left[b^2k^2\cos^2 k(x+V_w t)-\dfrac{b^2k^2}{2a^2}\sin 2k(x+V_w t)\right]e^{-2kx/a}}{\left\{1+b^2k^2e^{-2kx/a}\left[\cos k(x+V_w t)+\dfrac{1}{a}\sin k(x+V_w t)\right]^2\right\}^{\frac{1}{2}}}\,dx, \qquad (2\,a)$$

and

$$J=\int_0^\infty\frac{1+2e^{-2kx/a}\,b^2k^2\left[\cos k(x+V_w t)+\dfrac{1}{a}\sin k(x+V_w t)\right]^2}{\left\{1+b^2k^2e^{-2kx/a}\left[\cos k(x+V_w t)+\dfrac{1}{a}\sin k(x+V_w t)\right]^2\right\}^{\frac{1}{2}}}\,dx.$$

Hence

$$\overline{V}_x=0, \qquad (3\,a)$$

since I is finite and J is infinite. This result is to be expected in this hypothetical case since the propulsive elements at the rear of the filament (in the neighbourhood of finite x) cannot propel the non-propulsive elements of filament at $x=$ infinity.

(ii) Consider a finite filament of n wave-lengths ($0 \leqslant x \leqslant 2\pi n/k$), the upper limit of the integrals I and J is $2\pi n/k$ instead of infinity. This formula is complicated for all values of bk, but if the assumption is made that bk is small (so that b^3k^3 can be neglected), then a formula is given which can be compared with that given by the theory of constant amplitude which is valid over the same set of values of bk.

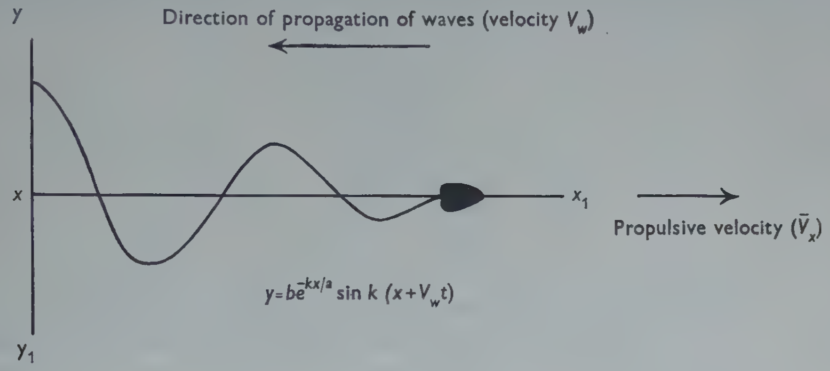

y
Direction of propagation of waves (velocity V_w)
x
x_1
Propulsive velocity ($\bar{V}_x$)
$y = be^{-kx/a} \sin k\,(x+V_w t)$
y_1

Fig. 2.

Equation (ii *a*), on the basis of this assumption, reduces to

$$\frac{\bar{V}_x}{V_w} = \tfrac{1}{2}b^2k^2(1-e^{-4\pi n/a})\left(\frac{a}{4\pi n} - \sin\frac{2kV_w t}{4\pi n}\right). \qquad \text{(iv } a\text{)}$$

This equation becomes

$$\frac{\bar{V}_x}{V_w} = \frac{b^2k^2}{2}$$

for no variation of amplitude (this follows by expanding the function $e^{-4\pi n/a}$ and letting $a \to \infty$) which is the standard formula for this case. Equation (iv *a*) also tends to zero as n, the number of wave-lengths, tend to infinity, which agrees with equation (iii *a*).

(iii) Taking the special case $a=4\pi$ then a table of the mean values of $\bar{V}_x/V_w$ depending on n the number of wave-lengths can be determined. Note that in this case the ratio of successive amplitudes between each wave-length is 0·6. For $a=4\pi$

n	$\bar{V}_x/V_w$,
1	$0{\cdot}32b^2k^2$,
2	$0{\cdot}22b^2k^2$,
3	$0{\cdot}16b^2k^2$.

It is interesting to note how the above values compare with the formula

$$\frac{\bar{V}_x}{V_w} = \tfrac{1}{2}b^2k^2,$$

where $\bar{b}$ is the mean amplitude of the motion, defined by

$$\bar{b}=\frac{1}{1+n}\sum_{a=0}^{a=n} b_a,$$

where b_a are the successive amplitudes after each wave-length, then

$$\bar{b}=\frac{b}{1+n}\left[\frac{1-0\cdot6^{n+1}}{0\cdot4}\right]$$

in the special case considered here. This approximation is equivalent to regarding the motion with varying amplitude as equivalent to that with the constant amplitude equal to the mean amplitude of the original motion. For $a=4\pi$

n	$\bar{V}_x/V_w=\frac{1}{2}\bar{b}^2k^2$,
1	$0\cdot32b^2k^2$,
2	$0\cdot22b^2k^2$
3	$0\cdot15b^2k^2$
∞	0.

It is seen that the correspondence between these two sets of results is very good, and gives an easy approximate method for obtaining the velocity of propulsion. This agreement is only valid for small values of *bk*, but it will be assumed that the same approximation of taking the mean amplitude will give reasonable results for all the larger values of *bk*.

SUMMARY

1. The general theory of flagellar propulsion is discussed and an expression obtained whereby the propulsive speed of a spermatozoon can be expressed in terms of the amplitude, wave-length and frequency of the waves passing down the tail of a spermatozoon of *Psammechinus miliaris*.

2. The expression obtained is applicable to waves of relatively large amplitude, and allowance is made for the presence of an inert head.

3. The calculated propulsive speed is almost identical with that derived from observational data. Unless the head of a spermatozoon is very much larger than that of *Psammechinus*, its presence makes relatively little difference to the propulsive speed. Most of the energy of the cell is used up in overcoming the tangential drag of the tail.

4. Although the amplitude may change as a wave passes along the tail, the propulsive properties of the latter may be expected to be closely similar to those of a tail generating waves of the same average amplitude.

REFERENCES

GRAY, J. (1953). Undulatory movement. *Quart. J. Micr. Sci.*, N.S., **94**, 551–78.

GRAY, J. (1955). The movement of sea-urchin spermatozoa. *J. Exp. Biol.* **32**, 775–801.

HANCOCK, G. J. (1953). The self-propulsion of microscopic organisms through liquids. *Proc. Roy. Soc.* A, **217**, 96–121.

TAYLOR, SIR GEOFFREY (1951). Analysis of the swimming of microscopic organisms. *Proc. Roy. Soc.* A, **209**, 447–61.

TAYLOR, SIR GEOFFREY (1952). The action of waving cylindrical tails in propelling microscopic organisms. *Proc. Roy. Soc.* A, **211**, 225–39.

FACILITATION IN SEA ANEMONES. IV. THE QUICK RESPONSE OF *CALLIACTIS PARASITICA* AT HIGH TEMPERATURES

By D. M. ROSS

Department of Zoology and Comparative Anatomy, University College, London

(*Received 27 June* 1955)

Preceding papers of this series have dealt with the effects of a number of treatments on the quick responses of the sea anemones *Calliactis parasitica* and *Metridium senile*. Pantin's (1935) analysis of the quick closing response in these animals showed that it is not normally elicited by single electrical stimuli, nor by stimuli following each other at intervals greater than about 3 sec. To get the closing response electrical stimuli must be given within a definite frequency range, at intervals of from about 0·2–2·5 sec. at room temperatures. Between these limits, the closer the stimuli the bigger the response, with responses occurring, of course, only to the second and subsequent stimuli. This remarkable example of a facilitated response in which the response can only occur when facilitation has been set up, and in which the amount of facilitation determines the size of the response, raised many interesting problems. My work has centred on the approach of trying to set up experimental conditions in which single stimuli are effective, as this seemed a promising way of finding out more about the nature of this kind of facilitation.

Few of the treatments employed so far have caused either *Metridium* or *Calliactis* to respond to single stimuli. Radical changes in ionic concentration do not do so, though they have big effects on the size of individual contractions (Ross & Pantin, 1940). A few drugs do so, e.g. tyramine, but only for brief periods after long exposure (Ross, 1945*a*). Some promising results with anemone extracts (Ross, 1945*b*) proved on further investigation to be unspecific effects shared with other substances (Ross, 1952) and were apparently due to a sensory rather than a direct facilitating action. Thus the aim of finding a chemical treatment which enables *Calliactis* or *Metridium* consistently to respond to single stimuli remains unfulfilled.

Recently I have found that if *Calliactis* is exposed to temperatures above 25° C., responses to single stimuli occur with great regularity. The responses obtained are generally small and might be regarded as insignificant were it not that so many other treatments have failed to produce this kind of effect. It seemed desirable, therefore, to study this effect as closely as possible and to report it briefly, since it reveals an unsuspected property of the quick response in these animals.

RESULTS

In these experiments the temperature of the sea water bathing the anemone was raised slowly by means of a small aquarium heater with thermostat attachment, the water being circulated by vigorous aeration. The animal was stimulated as required by condenser discharges delivered through fluid electrodes as in Pantin's (1935) original experiments. The experiments were carried out in London on animals obtained from Plymouth.

As the temperature rises the responses to stimuli become smaller and smaller at frequencies within the range effective at room temperatures. This is, of course, the effect of temperature on the rate of decay of facilitation described by Hall & Pantin (1937) in *Metridium*. Above 25° C., however, one begins to see tiny movements around the margin of the disk occurring in response to single stimuli. As the temperature rises still further these tiny movements become bigger and they have the effect of lifting all the tentacles quite sharply. When this happens it becomes clear that one is witnessing genuine withdrawal movements of the marginal sphincter that are similar in character and dimensions to the responses one gets normally to the second of a pair of stimuli as one approaches the end of the period of facilitation. At this point and beyond it is possible to record these movements on smoked paper.

At some point, usually between 30° and 34° C. in different animals, *Calliactis* becomes quite insensitive to electrical or tactile stimulation. It is possible, therefore, to follow the development of these responses to single stimuli only over a short temperature range of 5–9°.

Metridium does not behave like *Calliactis* in this respect. Hall & Pantin (1937) reported that temperatures above 25° C. were fatal to *Metridium*. In my experience, *Metridium* will recover temporarily from a temperature as high as 29° C., and death occurs some days later. Above 25° C., however, *Metridium* remains so tightly closed that its responses to stimulation can no longer be recorded or clearly observed. *Calliactis*, on the other hand, shows no ill effects from the treatment. Recovery of excitability occurs quickly as the temperature falls to a normal level, even when it has been exposed to temperatures as high as 35° C. It is noteworthy that as the temperature falls again the first responses to reappear are the small responses to single stimuli, usually in the neighbourhood of 30° C. Animals may be subjected to this treatment time and again without any apparent change in their normal behaviour or in their response to stimulation.

Figs. 1–3 show records of the responses of *Calliactis* to stimuli at different frequencies at temperatures ranging from room temperatures up to 33·5° C. In Fig. 1 we see responses at a frequency of 1 stimulus per second. At a temperature of 19·5° C. the steps of the staircase are big and the response begins, of course, only on the second stimulus, so that the five stimuli give a staircase consisting of four big steps. At 25° C. the response still begins on the second stimulus, but the four steps of the staircase are now quite tiny, as Hall & Pantin (1937) showed with *Metridium*. With a further rise in temperature, however, the response takes on a different character. A small contraction can be seen on the first stimulus, and usually on all subsequent stimuli, so that the five stimuli now give a staircase with five small steps instead of four, and the response is an example of simple summation. At these temperatures the quick response of *Calliactis* clearly loses its normal facilitated character, and responses occur to each stimulus without requiring a preceding facilitating stimulus.

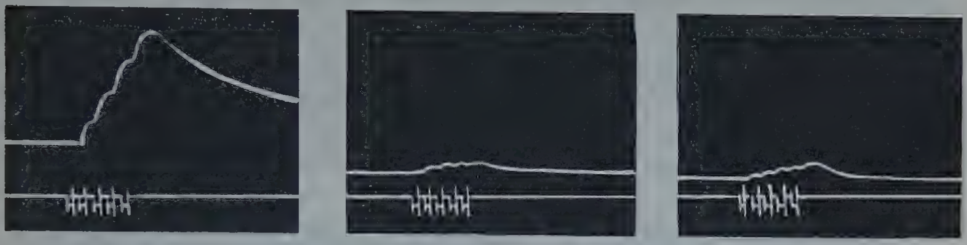

19·0° 25·0° 29·5°

Fig. 1. Responses of *Calliactis parasitica* to 5 stimuli at 1 per second at the temperatures indicated.

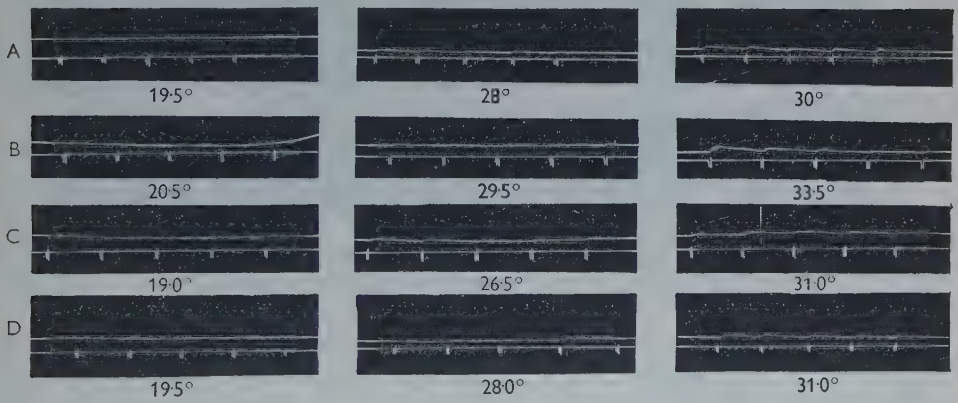


Fig. 2. Responses of *Calliactis parasitica* to 5 stimuli at 1 per 10 seconds at the temperatures indicated. A, B, C & D are four different experiments.

Fig. 2 shows the same effect in another way. The stimuli here are delivered at intervals of 10 sec. This is well outside the normal period of decay of facilitation, so that each stimulus is in effect a single stimulus. These stimuli have no effect at room temperatures, but as the temperature rises to 30° C. and beyond, each stimulus elicits a distinct movement of the animal. These responses are never large, and their size varies considerably from one animal to another, as the records taken from four different experiments show. In my experience the responses given by any one animal to the separate stimuli in a series are usually all approximately the same size at a given temperature, as in Figs. 2*a*, *c* and *d*, but occasionally bigger responses occur on certain stimuli as in Fig. 2*b*.

It is evident from these records that these movements in response to single stimuli get bigger as the temperature rises. This feature of the effect is sometimes easier to see by eye, as a lifting of the tentacles which becomes more and more pronounced, than it is to record on a kymograph. This heightening and sharpening of the response continues until the temperature is reached at which the animal becomes inexcitable. One gets the impression that the response would get still bigger and would eventually approach the size of the normal quick closing movements if *Calliactis* could tolerate still higher temperatures.

Fig. 3 shows the responses of *Calliactis* to pairs of stimuli at intervals of 0·5, 0·3 and 0·2 sec. We see that at the highest temperatures employed, 31–32° C., the decay of facilitation is so speeded up that only the small responses to single stimuli are apparent at 0·5 sec. The existence of these responses makes it impossible to determine the period of decay of facilitation with any precision because it is no longer clear at what frequency the response to the second stimulus of a pair fades out when every stimulus gives a small response. At higher frequencies, however, the normal type of facilitated response to the second stimulus is superimposed on, and at 0·2 sec. quite overshadows, the response to the first stimulus (Fig. 3*c*, 31·5° C.).

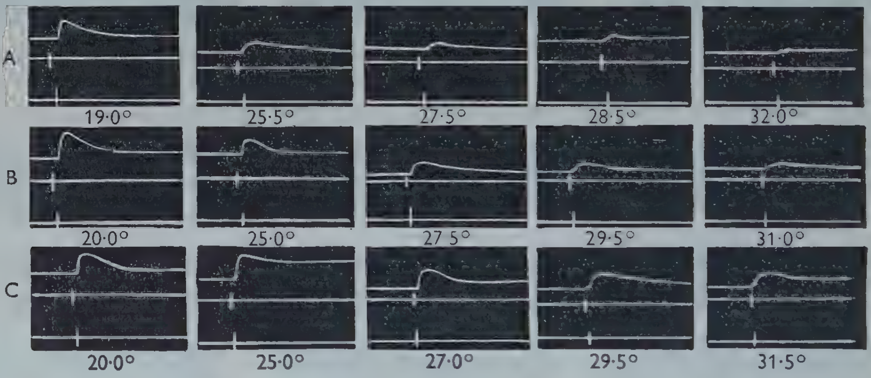


Fig. 3. Responses of *Calliactis parasitica* to pairs of stimuli at frequencies of: (A) 0·5 sec.; (B) 0·3 sec.; (C) 0·2 sec. at the temperatures indicated.

DISCUSSION

Earlier work has produced abundant evidence that facilitation and excitation are separate steps in transmission of the quick response in *Calliactis* (Pantin, 1937; Ross & Pantin, 1940; Ross, 1945). Within the framework of this general conception it is possible to put forward a number of possible explanations for the temperature effect here described. It is conceivable that at high temperatures a slight state of permanent facilitation might be set up perhaps as a result of the metabolic effects of the treatment. This is not, however, an example of the residual facilitation described by Ross & Pantin (1940) in *Calliactis* and by Pantin & Vianna Dias (1952) in a Brazilian species of *Bunodactis*. That effect occurs only after repeated stimulation, whereas the responses to single stimuli described here occur in the absence of previous stimulation. It is also possible that this treatment so affects the electrical conditions at the membrane of the muscle cells or at the junctions that the normally ineffective single impulse is just able to initiate contractions without prior facilitation. Or perhaps at these temperatures there are enough random impulses passing in the nerve net to keep all the junctions slightly facilitated. Yet if the treatment really had effects on the electrical state of the muscle or the junctions, or if it set up numbers of impulses, one would expect a conspicuous increase in the number of spontaneous quick contractions. This does not happen.

We are in no position to discuss these possibilities further, since they are concerned with aspects of neuromuscular activity on which we have little or no information at present in these animals. I propose, however, to discuss one other possibility in more detail, partly because it arises from the idea which led me to undertake these experiments. This is that the puzzling failure of the single stimulus in *Calliactis* and other anemones may simply be a matter of timing between the facilitatory and excitatory steps in neuromuscular transmission in these animals.

Suppose that in this response, where facilitatory and excitatory processes are both essential for transmission, facilitation is a slower process than excitation. In that case a single stimulus might fail simply because the excitatory state which starts off the quick contraction has subsided before any facilitation has been set up. Now if facilitation could be speeded up more than excitation, say by raising the temperature, so that some facilitation is set up before the excitatory state has died away, one should get responses to single stimuli as the two processes begin to overlap in time.

One merit of this approach is that, unlike the suggestions made above, it postulates only that the effect of raising the temperature is to speed up reactions. There is, of course, no direct evidence that facilitation is established more quickly at higher temperatures. But it decays more quickly and the whole frequency/response curve is shifted, so it would be surprising if the build-up of facilitation were not affected in the same way.

The notion that facilitation might be speeded up more than the process of excitation is less plausible and would require some experimental support. Although the junctional and muscle excitation time cannot be measured directly, there are two quantitative features of the quick response that might give some indication about the effect of higher temperatures on excitatory processes in general. The latent period, the time between the application of a successful stimulus and the beginning of the contraction comprises the conduction time of the impulse in the nerve net and the excitation time at the muscle, the latter being the property which one would like to measure. But both of these components no doubt are determined by processes involving movements of ions at membranes, and the effects of temperature on them might reasonably be expected to be of the same order of magnitude. The absolute refractory period is the shortest interval between a pair of stimuli that still permits a response to occur on the second. It is thus a measure of the restoration of excitability and should also indicate how higher temperatures affect the excitatory process from which the system is recovering during this period.

With this in mind I compared the effects of higher temperatures on the three quantities, rate of decay of facilitation, absolute refractory period, and latent period in several animals. The results are shown in Fig. 4. There are certain difficulties in measuring these quantities exactly at temperatures above 28° C. (especially the decay of facilitation, owing to the appearance of responses to single stimuli), but the trend is decidedly in favour of a much bigger temperature effect on facilitation than on the other two quantities. This is true particularly above 23° C., where latent and refractory periods show little change. This result is consistent with the idea advanced above, that at higher temperatures facilitation might begin to overtake the excitatory process. Certainly if this were happening one would expect the effect to begin with tiny responses which gradually get bigger as the two processes overlap more and more. This would be more convincing, of course, if the responses obtained at the highest temperatures were not so small. In this connexion it would be interesting to repeat these tests on *Calliactis* from warmer waters, since they might tolerate still higher temperatures and so allow the effect to be studied over a wider range.

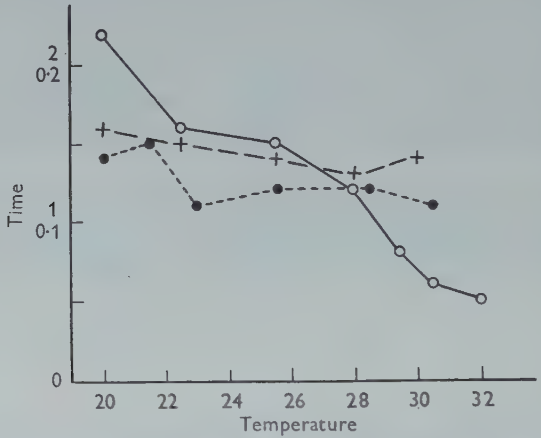


Fig. 4. Effect of temperature on Facilitation, Refractory Period and Latent Period in the quick response of *Calliactis parasitica*. —○— Duration of facilitation, (seconds). - - + - - Absolute refractory period (tenths of seconds). - - ● - - Latent period (tenths of seconds).

A comment might be added about the bearing these observations have on comparative aspects of neuromuscular transmission, and especially on the relationship between facilitated and direct modes of transmission. The records in Fig. 1 show that at high temperatures *Calliactis* goes over from a facilitated to a direct mode of transmission with simple summation, though it is direct transmission of a very imperfect and inefficient kind. Whether this happens by speeding up the facilitation process so that it begins to overtake the more rapid excitation process as I have suggested, or in some other way, it does show that the distinction between the two modes of transmission is not absolute. This encourages the belief that it will yet be possible to solve the elusive problem of fitting the facilitation process in anemones into more general concepts of neuromuscular transmission which have been found to apply in most other animals.

SUMMARY

1. At temperatures above 25° C. small quick contractions of the sphincter of the sea anemone, *Calliactis parasitica*, occur in response to single electrical stimuli which are normally ineffective.

2. These contractions, though they never become large, increase in size as the temperature rises until they vanish, along with all other responses, between 30 and 35° C.

3. The effect is discussed in relation to the timing of the two processes, facilitation and excitation, involved in the quick response in this animal.

REFERENCES

HALL, D. M. & PANTIN, C. F. A. (1937). The nerve net of the Actinozoa. V. Temperature and facilitation in *Metridium senile*. *J. Exp. Biol.* **14**, 71–8.

PANTIN, C. F. A. (1935). The nerve net of the Actinozoa. I. Facilitation. *J. Exp. Biol.* **12**, 119–38.

PANTIN, C. F. A. (1937). Discussion meeting on the transmission of excitation in living material. Junctional transmission of stimuli in the lower animals. *Proc. Roy. Soc.* B, **123**, 397–9.

PANTIN, C. F. A. & VIANNA DIAS, M. (1952). Excitation phenomena in an Actinian (*Bunodactis* sp.?) from Guanabara Bay. *Ann. Acad. bras. Sci.* **24**, 335–49.

ROSS, D. M. (1945*a*). Facilitation in sea anemones. I. The action of drugs. *J. Exp. Biol.* **22**, 21–31.

ROSS, D. M. (1945*b*). Facilitation in sea anemones. II. Tests on extracts. *J. Exp. Biol.* **22**, 32–6.

ROSS, D. M. (1952). Facilitation in sea anemones. III. Quick responses to single stimuli in *Metridium senile*. *J. Exp. Biol.* **29**, 235–54.

ROSS, D. M. & PANTIN, C. F. A. (1940). Factors influencing facilitation in Actinozoa. The action of certain ions. *J. Exp. Biol.* **17**, 61–73.

ELECTROMETRIC TITRATION OF CHLORIDE IN SMALL VOLUMES

By J. A. RAMSAY, R. H. J. BROWN AND P. C. CROGHAN
Zoological Laboratory, University of Cambridge

(*Received 27 July* 1955)

The potentiometric end-point in the titration of chloride with silver nitrate depends upon the fact that the concentration of Cl^- ions determines the potential of a silver wire coated with silver chloride which dips into the titration vessel. This chloride electrode is connected through a voltmeter with a reference electrode which completes the circuit, and when the end-point is reached there is a very rapid change of potential between the electrodes, of the order of 100 mV or more. This method is not only convenient but also promises to be practicable on the micro-scale where there is insufficient thickness of solution for the colour change of an indicator to be perceptible.

Advances in the field of potentiometric titration have been frequently reviewed (see, for example, Furman, 1954). The possibilities of the potentiometric end-point in microanalysis were first exploited by Schwarz & Schlösser (1933), who were able to measure 1 μg. of chloride with an accuracy of 'a few per cent'. Linderström-Lang, Palmer & Holter (1935) reduced the scale of operation to 0·4 μg. with an error of ± 4·5 %. Cunningham, Kirk & Brooks (1941) measured 0·5 μg to ± 2 %. Since the present work was first taken in hand, Shaw (1955) has published a method accurate to ± 1 % in less than 1 μg.

While the principle of the method is the same in all cases the form of apparatus used has been very different, particularly in respect of the titration vessel and the reference electrode. Schwarz & Schlösser used a small flask and an $Ag/AgNO_3$ non-polarizable electrode. Cunningham *et al.* titrated in an open dish using a silver-mercury amalgam wire as reference electrode. Linderström-Lang *et al.* used a silver wire sealed into the tip of the burette, and a similar arrangement was also adopted by Sanderson (1952) and by Shaw, the latter carrying out the titration in a drop resting upon a hydrofuge surface.

In the course of this work we have developed two methods. The first is on the 1 μg. scale and has much the same accuracy as Shaw's method, with a possible slight advantage in the form of the burette used. The second method operates on the scale of 10^{-3} μg. (or, the scale of 10^{-3} μl.) and introduces a new feature in that the Ag^+ ions are added not as a solution from a burette but by electrolysis of the silver electrode, the quantity of electricity passing being the measure of the chloride present.

THE FIRST METHOD

The apparatus required for the first method is illustrated in Fig. 1. A Scholander-type burette is used, containing 0·01 N-$AgNO_3$, one unit on the micrometer head corresponding to 0·078 μl. A platinum wire is sealed into the delivery limb. The titration vessel is a small Pyrex tube 0·5 cm. in internal diameter and 2 cm. long, carried upon a platform which can be raised so that the delivery limb of the burette dips into and supports the titration vessel. Also dipping into the titration vessel is the silver wire electrode and a silica capillary from which compressed air is bubbled to stir the contents of the vessel. The voltmeter is similar in all but minor detail to that described by Sanderson but has a higher input impedance of 10 MΩ.

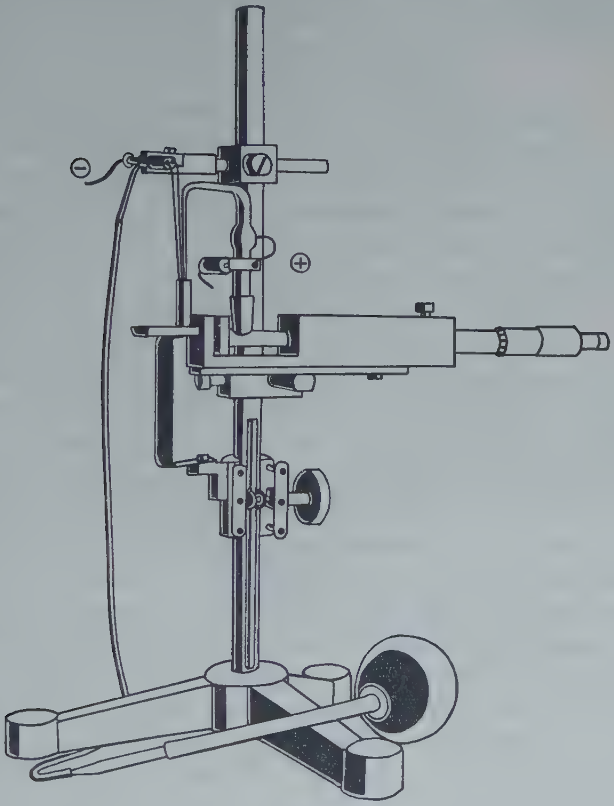


Fig. 1.

About 20 μl. of 50% acetic acid are first measured into the titration vessel. The sample is taken up in a micropipette and washed out with the acetic acid in the vessel. The vessel is then placed in position and the burette is read. The voltmeter is adjusted so that the needle is near the top of the scale. As the end-point is approached the silver nitrate is added in steps of one unit. After each step the contents of the titration vessel are thoroughly mixed and the potential is recorded. This is continued until the end-point has been passed. Voltmeter reading can be plotted against burette reading and the end-point is given by the point of inflexion of the curve. At the end of the titration the platform is lowered, the titration vessel is removed and the dipping assembly is washed with a jet of distilled water. The burette is so mounted that it can be rotated about the axis of the stand and is thus separated from the rest of the assembly for purposes of refilling. A single determination can be completed in 5 min.

The method has been tested with pure NaCl solutions and with biological fluids ranging from 0·2 to 10 μl. in volume and from 10 to 500 m.equiv./l in chloride concentration; the standard deviation of a series of similar titrations is less than $\pm 1\%$. Sanderson has shown that protein does not interfere with the potentiometric end-point and that no error is introduced by the presence of phosphate, sulphate or oxalate. To this list it has been possible to add citrate and benzene-sulphonate.

THE SECOND METHOD

The possibility of reducing the scale of the potentiometric titration is limited by two factors: (*a*) the deterioration in the sharpness of the end-point as the dilution is increased, and (*b*) the increase in volumetric error as the volumes of sample and titrant are reduced. Schwarz & Schlösser pointed out that the sharpness of the end-point depends upon the solubility product of silver chloride and showed that the sharpness could be improved in the presence of high concentrations of alcohol or acetone; in this way they were able to work with solutions as dilute as 10^{-4} N. In biological work, however, the dilution factor need not be serious, since the body fluids of animals have chloride concentrations in the range 10–500 m.equiv./l., for which an adequately sharp end-point can be expected. It is more usually the small quantity of fluid available which calls for reduction in scale. A technique has already been developed (Ramsay, 1950) whereby small volumes of fluid are kept under liquid paraffin and from which samples can be drawn up to a mark in a fine silica pipette. Although the volume of the pipette—of the order of 10^{-3} μl.—is only known approximately, the volumes delivered are reproducible to $\pm 3\%$ or better. Given some means of measuring the length of the column in the silica tube, e.g. a travelling microscope, it is clear that this technique forms a basis for an attempt to reduce the scale of the method by about one thousand-fold. In some early attempts the sample was deposited from one pipette upon the end of a fine silver wire, under liquid paraffin, and contact was made between it and a reference electrode through a fine capillary. Another pipette was filled with silver nitrate and used as a burette. Titrations carried out in this way were reproducible within $\pm 2{\cdot}5\%$ (standard deviation). But while this method was being developed it occurred to us that as an alternative to adding silver nitrate from a burette it would be possible to liberate Ag^+ ions by passing a current through the system and that this current could be used to charge a condenser whose voltage would then be a measure of the amount of Ag^+ ion liberated.

Consider the following system:

$$\mathrm{Ag} \mid 0{\cdot}1\,\mathrm{N\text{-}AgNO_3} \;\vdots\; 0{\cdot}1\,\mathrm{N\text{-}NaNO_3} \;\vdots\; \underset{\text{(small volume)}}{0{\cdot}1\,\mathrm{N\text{-}NaCl}} \mid \mathrm{AgCl/Ag}.$$

Since silver can act as a reversible electrode, not only to Ag^+ ions but also (when coated with silver chloride) to Cl^- ions, the system forms a concentration cell which (liquid junction potentials disregarded) is capable of developing a potential of about 450 mV.

When the two silver electrodes are connected through an external circuit current will flow and Ag^+ ions will be liberated from the Ag/AgCl electrode until their concentration around it reaches 0·1 N. The first Ag^+ ions to be liberated will combine with the Cl^- ions of the 0·1 N-NaCl, and when all the Cl^- ions have been precipitated the potential will have fallen to about 225 mV. If a condenser is included in the external circuit the titration current will develop a voltage across it. For convenience we will refer all calculations to a 'standard sample', $10^{-3}\,\mu$l. of 0·1 N-NaCl. The quantity of electricity passed during titration of the standard sample is $9{\cdot}65 \times 10^{-6}$ coulomb. The voltage across the condenser cannot be greater than the voltage given by the concentration cell; at the end-point this is 225 mV., from which it can be calculated that the capacity of the condenser must be at least 43 μF.

In practice this arrangement would have the disadvantage that with the effective titration potential falling to zero the approach to the end-point would be intolerably slow. This can be overcome either (*a*) by increasing the capacity of the condenser or (*b*) by including an additional source of potential in the circuit. The capacity may be increased either directly by adding more condensers, which would inconveniently increase the size of the apparatus, or indirectly by electronic devices which would need very carefully stabilized supplies. The stepping-up of potential is not without its dangers, since if the potential across the cell exceeds 1·7 V. there may be electrolysis of water as well as of silver. We have compromised by including a 1·5 V. dry battery in the circuit, which has made it possible to reduce the condenser bank to 20 μF.

Another important matter is the current taken by the voltmeter and the leakage currents of the whole system, considered in relation to the quantity of electricity involved and the time taken to complete the titration. The quantity of electricity is $9{\cdot}65 \times 10^{-6}$ coulomb, and a reasonable estimate of the titration time is 2 min. For the error to be within 1 % the leakage must not exceed $9{\cdot}65 \times 10^{-8}$ coulomb in 2 min., which corresponds to a current of 8×10^{-10} amp. The grid current of electrometer valves is of the order of 10^{-12} amp. or less, but the leakage current of the condenser bank approaches the critical value. The condensers used are ordinary radio components, 2 and 4 μF., 800 V, d.c. working, specially selected for high leakage resistance. Measurements made on these condensers indicate a leakage current of $0{\cdot}81 \times 10^{-10}$ amp./μF./V. If the capacity is 20 μF., the voltage developed in titration of the standard sample will be 487 mV., and under these conditions the leakage current will be $7{\cdot}9 \times 10^{-10}$ amp. Special condensers with plastic insulation, having a leakage resistance about one hundred times higher than paper condensers, are prohibitively expensive.

The details of the apparatus are shown in Fig. 2. A layer of silicone D.C. 1107 is deposited upon the silver wire and a clean surface is produced by cutting off the end. The clean surface is wettable, whereas the silicone-covered surface is non-wettable; the droplet is therefore stable when in contact with the end of the wire. Contact is made between the sample and the reference electrode through a fine glass capillary. From its opening, about 20 μ in diameter, the capillary rapidly widens to about 1 mm. internal diameter. This part of the system is filled with N-$NaNO_3$ set in agar; it continues into a vertical tube containing N-$NaNO_3$ (without agar) under a layer of liquid paraffin. A similar vertical tube contains a silver wire in N-$AgNO_3$ under liquid paraffin, and contact is made between the two tubes with a N-$NaNO_3$-agar bridge. The reason for this complication is that the penetration of Ag^+ ions into an agar gel results in the darkening of the gel; with the arrangement just described this penetration is limited to the bridge which is discarded and replaced when necessary. The resistance of the reference electrode system is of the order of 7 MΩ.

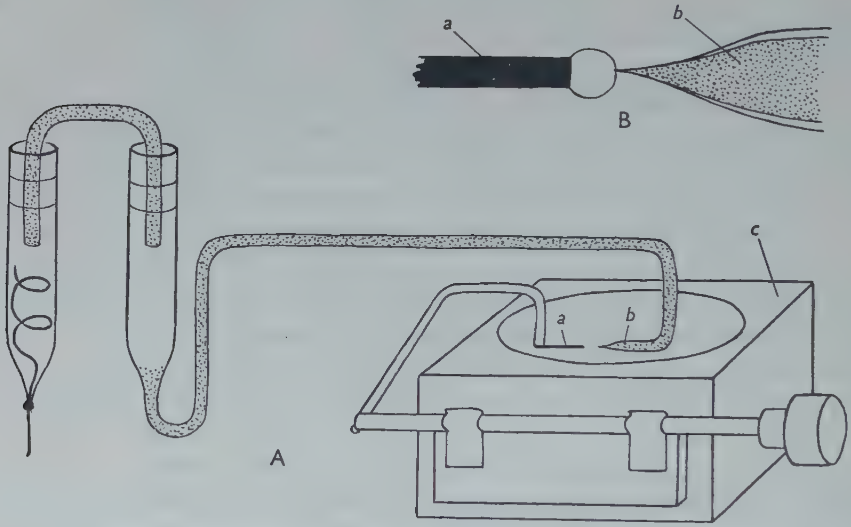


Fig. 2. A, general arrangement. B, silver wire, sample and reference electrode enlarged. *a*, silver wire electrode; *b*, capillary of reference electrode; *c*, Perspex bath.

The liquid paraffin in which the sample is submerged is contained in a Perspex bath mounted upon a small table about 5 in. above the bench. This makes it possible to place a binocular microscope in position to view the sample with transmitted light. The brass arm carrying the silver wire is friction-filled to the side of the bath and provides for up and down movement (by rotation) and for movement from left to right (by sliding). Adjustment in the fore and aft direction is provided for on the mounting of the reference electrode; but once this has been correctly positioned it can be left while the silver electrode is removed for cleaning and replaced after each titration.

N-H_2SO_4 is used instead of acetic acid since the latter is soluble in liquid paraffin. This acid is first drawn up to the mark in the silica pipette, a small length of liquid paraffin is drawn in and then the sample of chloride-containing solution is drawn up to the mark. The tip of the pipette is brought up to the silver wire and its contents are driven out. It is convenient to bring up the pipette upon the right-hand side, and for this reason the silver wire is directed to the right. The reference electrode must be mounted upon the left so as to clear the pipette. These requirements account for the somewhat twisted aspect of the arrangement shown in Fig. 2*b*.

The electrical circuit is shown diagrammatically in Fig. 3. Switches provide for nominal capacities of 4, 8, 12, 16 and 20 μF. and for series resistances of 1, 6, 11 and 21 MΩ. When the button is pressed the titration current is switched on, and when it is released the cell is connected to the voltmeter. The condenser-bank shorting switch is operated by lifting and turning the press-button. In view of the low voltages involved all contacts are made of silver and have a slight wiping action.

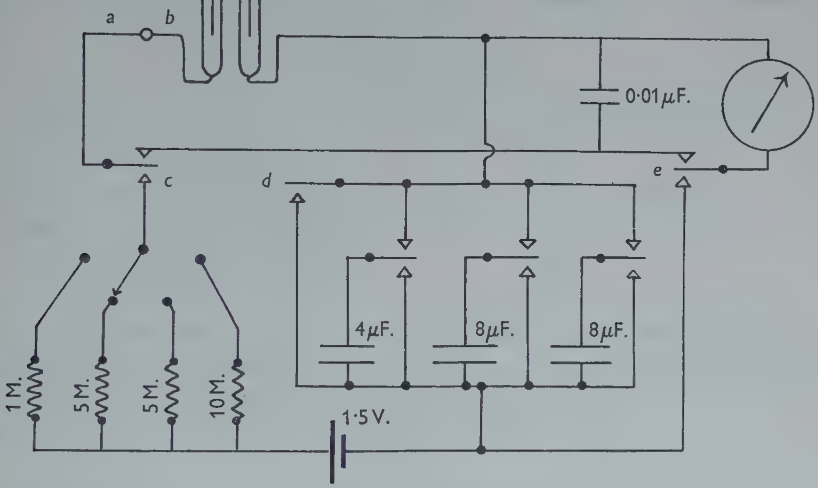


Fig. 3. Circuit diagram. *a*, silver wire electrode; *b*, reference electrode; *c*, titration button; *d*, condenser shorting switch; *e*, voltmeter switch.

They are mounted upon Perspex to ensure a high leakage resistance. The 0·01 μF. condenser across the leads from the cell to the voltmeter serves to stabilize the instrument while it is open-circuited during the passage of the titration current.

For the voltmeter an instrument with a galvanometer scale reading directly in millivolts is to be preferred to one in which the galvanometer is used for null-point only. We have made use of a Pye Universal pH Meter, taking a current of 10^{-12} amp.

Since the Ag^+ ion cannot be added in small steps of equal size (owing to variations in electrical resistance and effective potential), the end-point cannot be conveniently ascertained by the graphical method. Instead, it is necessary to titrate to a definite potential which can be arbitrarily chosen provided that it lies in the steep part of the curve; in the present work a potential of 240 mV. has been taken as the end-point. The potential registered at the beginning of the titration is a useful indicator of the concentration of chloride present, e.g.

100 m.equiv./l. approx. 450 mV.,
10 m.equiv./l. approx. 390 mV.,
1 m.equiv./l. approx. 330 MV.

The series resistance can be adjusted to the operator's taste as the titration proceeds. The titration button is pressed for a few seconds at a time, and after it is released the galvanometer pointer falls abruptly, rising to a steady value in 2–3 sec. When the end-point is reached the voltmeter is switched to the condenser bank and the voltage is recorded. The silver wire is then removed, washed with a jet of distilled water and replaced. When the apparatus is not in use the silver wire is replaced with a platinum wire loop containing a drop of N-$NaNO_3$, into which the end of the capillary is inserted.

Table 1

Volume of sample (µl. × 10^{-3})	Concentration (m.equiv./l.)	Quantity (µg. × 10^{-3})	Capacity (nominal) (µF.)	Voltmeter readings (mV.)	Mean (mV.)	Standard deviation (%)
1·64	20	1·16	4	777, 780, 776, 775, 773	776	±0·33
1·64	5	0·29	4	201, 199, 201, 201, 202	201	±0·55
1·64	1	0·08	1	161, 160, 170, 166, 157	163	±3·3
0·545	100	1·93	8	688, 704, 686, 701, 693, 688, 694, 694, 696, 691	693·5	±0·84
0·145	100	0·51	4	344, 356, 364, 370, 355	358	±2·85

A representative set of data is presented in Table 1. From this table it can be seen that the standard deviation is < ±1 %, provided that the volume of the sample is not less than $0·545 \times 10^{-3}$ µl. and that its concentration is not less than 5 m.equiv./l.; that is to say, the method will measure 10^{-4} µg. of chloride with a standard deviation of < ±1 %.

The relation between amount of chloride and condenser charge is found to be linear, as expected from theory.

The volume of the silica pipette has hitherto been determined from measurements of its length and diameter made with an eyepiece micrometer, no allowance being made for refraction or for departure from circular cross-section. The measurement of diameter is not believed to be better than ±10 %, which implies a volume error of ±20 %. The method was therefore first envisaged as a comparative one, in which the titre of an unknown sample would be compared with the titre of a sample of known solution taken with the same pipette. The success of the method and the general agreement with theory embolden us to believe that the charge on the condenser is in fact an absolute measure of the amount of chloride titrated. If this is true, we have an effective means of determining the volume of the pipette, since the electrical measurements can be carried out with some precision. The volumes quoted in Table 1 have been arrived at in this way.

As already mentioned it has been shown that there is no error due to the presence of protein when the titration is carried out on the macro-scale, and there is no reason to believe that protein will affect the electrolytric titration just described. This was checked nevertheless by comparison of human serum with 100 mM/l. NaCl, using both first and second methods, and agreement was found to within the expected limits of error. The presence of protein does affect the second method, however, in the following way. After some half dozen titrations of protein-containing samples it is observed that the resistance of the system has increased, and that when the titration button is released the galvanometer pointer does not swing up quickly and smoothly to its new value, but rises irregularly over a period of several seconds. The symptom is such as would be produced by a barrier to diffusion between the surface of the electrode and the body of the sample, and if the cake of silver chloride is dislodged the course of titration returns to normal at once. The same symptom appears in the absence of protein if the cake of silver chloride in the electrode is allowed to dry in air. In all these cases, of course, the remedy is to produce a clean surface of silver by a fresh cut.

SUMMARY

1. Two methods of titrating chloride with Ag^+ ion using the potentiometric end-point are described.

2. The first method is conventional in that silver nitrate is added from a burette. It deals with volumes down to 0·2 μl. and can measure 1 μg. of chloride with an error of $< \pm 1\%$ (standard deviation).

3. According to the second method Ag^+ ion is added by passing a current through a silver electrode in series with a condenser. The charge developed on the condenser is a measure of the amount of chloride titrated. This method deals with volumes down to $0{\cdot}5 \times 10^{-3}$ μl. and can measure 10^{-4} μg. of chloride with an error of $< \pm 1\%$ (standard deviation).

4. As far as is known these methods are not susceptible to interference from other substances likely to be present in biological fluids.

We wish to record our thanks to Dr J. N. Agar and Dr R. N. Haszeldine for their comments upon the first draft of this paper.

REFERENCES

CUNNINGHAM, B., KIRK, P. L. & BROOKS, S. C. (1941). Quantitative drop analysis. XIV. Potentiometric determination of chloride. *J. Biol. Chem.* **139**, 11–19.

FURMAN, N. H. (1954). Potentiometric titrations. *Analyt. Chem.* **26**, 84–90.

LINDERSTRÖM-LANG, K., PALMER, A. H. & HOLTER, H. (1935). Studies on enzymatic histochemistry. XIV. A microdetermination of chloride in tissues. *C.R. Lab. Carlsberg*, Ser. Chim., **21**, 1–5.

RAMSAY, J. A. (1950). The determination of sodium in small volumes of fluid by flame photometry. *J. Exp. Biol.* **27**, 407–19.

SANDERSON, P. H. (1952). Potentiometric determination of chloride in biological fluids. *Biochem. J.* **52**, 502–5.

SCHWARZ, K. & SCHLÖSSER, C. (1933). Die potentiometrische Bestimmung kleinster Chloridmengen. *Mikrochemie*, **13** (N.F. 7), 18–30.

SHAW, J. (1955). A simple procedure for the study of ionic regulation in small animals. *J. Exp. Biol.* **32**, 321–9.

INDEX OF SUBJECTS

A

Allolobophora chlorotica Savigny:
See Earthworms
Ammonia:
See Sialis lutaria
Antennal glands:
See Palaemonetes varians (Leach)
Anodonta cygnea L.
The behaviour of, and its neurophysiological basis (Barnes) 158
Arachnids:
See Lyriform organs

B

Barnacle cyprids:
The behaviour of, in relation to water movement over a surface (Crisp) 569
Behaviour:
of *Anodonta cygnea* L., and its neurophysiological basis (Barnes) 158
— of barnacle cyprids in relation to water movement over a surface (Crisp) 569
Bird navigation:
An investigation of the 'chronometer' factor in (Matthews) 39
Blood groups:
of the rabbit, a study of, with reference to the inheritance of three antigens and the agglutinability of the red cells carrying them (Joysey) 440
Brook lamprey:
Photo-kinesis in the ammocoete larva of (Harden Jones) 492

C

Calliactis parasitica:
See Sea anemones
Calliphora:
See Ocelli
Calliphora erythrocephala Meig.:
Oxygen consumption of castrated females of the blow-fly (Thomsen and Hamburger) 692
Carcinus maenas:
Ionic regulation in the muscle fibres of. I. The electrolyte composition of single fibres (Shaw) 383
— —II. The effect of reduced blood concentration (Shaw) 664
Cell division:
The mechanism of, in the cleavage of the newt's egg (Selman and Waddington) 700
Cell surface:
The mechanical properties of. III. The sea-urchin egg from fertilization to cleavage (Mitchison and Swann) 734
Chloride:
Electrometric titration of, in small volumes (Ramsay, Brown and Croghan) 822
Cockroach:
Wax secretion in (Beament) 514
Cucumaria:
See Echinodermata
Cuticle(s):
The permeability and structure of, of the aquatic larva of *Sialis lutaria*, (Shaw) 330
— The wetting of insect, by water (Holdgate) 591
— *See also* Honey-bee

D

Dixippus morosus:
The excretion of sodium, potassium and water by the Malpighian tubules of the stick insect (Orthoptera, Phasmidae) (Ramsay) 200
— The excretory system of the stick insect (Orthoptera, Phasmidae) (Ramsay) 183

E

Earthworms:
The water relations of. I. The activity of the nephridiostrome cilia of *Lumbricus terrestris* L. and *Allolobophora chlorotica* Savigny, in relation to the concentration of the bathing medium (Roots) 765
Echinodermata:
Studies on the myoneural physiology of. II. Cricumoral conduction in Circumaria (Pople and Ewer). 59
Egg:
See Newt's egg, Sea-urchin egg *and* Cell surface
Excretion:
of sodium, potassium and water by the Malpighian tubules of the stick insect, *Dixippus morosus* (Orthoptera, Phasmidae) (Ramsay) 200
— and storage of ammonia by the aquatic larva of *Sialis lutaria* (Neuroptera) (Staddon) 84
Excretory system:
of the stick insect, *Dixippus morosus* (Opthoptera, Phasmidae) (Ramsay) 183

F

Flight:
in mosquitoes, the sources of energy for (Clements) 547
Frog:
See Leptodactylus ocellatus

G

Geryonia proboscidalis:
See Medusae
Growth and moulting:
See Rhodnius prolixus

H

Haemocytes:
The role of the, in the growth and moulting of an insect, *Rhodnius prolixus* (Hemiptera) (WIGGLESWORTH) 649
Haemoglobin:
See Nephthys
Homing ability:
An investigation of, in pigeons without previous homing experience (PRATT) 70
Homing orientation:
in pigeons in relation to opportunity to observe the sun before release (PRATT and THOULESS) 140
Honey-bee:
The cuticular waterproofing mechanism of the worker (GLYNNE JONES) 95
Hydra:
See Osmoregulation

I

Insect cuticles:
See Cuticle(s)
Insect ocellar nerve:
Functioning of (HOYLE) 397
Insect tracheal system:
Further studies of gas-filling in (BUCK and KEISTER) 681
Insects:
See Temperature excess
Ionic regulation:
in the muscle fibres of *Carcinus maenas*. I. The electrolyte composition of single fibres (SHAW) 383
— — II. The effect of reduced blood concentration (SHAW) 664
— osmoregulation and, in *Hydra* (LILLY) 423
— A simple procedure for the study of, in small animals (SHAW) 321
— and water balance in the aquatic larva of *Sialis lutaria* (SHAW) 353
Isopods:
See Transpiration

L

Lamprey:
See Brook lamprey
Leptodactylus ocellatus:
Membrane potentials in the muscles of the South American frog (HARRIS and MARTINS-FERREIRA) 539
Light:
On the reaction to, of *Myxine glutinosa* L. (NEWTH and ROSS) 4
Light sense:
Experiments on, of the hag, *Myxine glutinosa* L. (STEVEN) 22
Locusta:
See Ocelli
Locusta migratoria migratorioides (R & F.):
Some temperature responses of nymphs of, with special reference to aggregation (CHAPMAN) 126
Lumbricus terrestris L.:
See Earthworms
Luminescence:
See Renilla
Lyriform organs:
The function of, of arachnids (PRINGLE) 270

M

Malpighian tubules:
See Dixippus morosus
Medusae:
The nerves and muscles of. II. *Geryonia proboscidalis* Eschscholtz (HORRIDGE) 555
— — III. A decrease in the refractory period following repeated stimulation of the muscle of *Rhizostoma pulmo* (HORRIDGE) 636
— — IV. Inhibition in *Aequorea forskalea* (HORRIDGE) 642
Membrane potentials:
See Leptodactylus ocellatus
Mosquitoes:
The sources of energy for flight in (CLEMENTS) 547
Muscles:
See Leptodactylus ocellatus, Medusae *and* Ionic regulation
Myoneural physiology:
See Echinodermata
Myxine glutinosa L.:
On the reaction to light of (NEWTH and ROSS) 4
— Experiments on the light sense of the hag (STEVEN) 22

N

Nephthys:
Observations on the respiratory physiology and on the haemoglobin of the polychaete genus, with special reference to *N. hombergii* (Aud. et M.-Edw.) (JONES) 110
Nerves:
See Medusae
Newt's egg:
The mechanism of cell division in the cleavage of (SELMAN and WADDINGTON) 700

O

Ocelli:
The functions of the, of *Calliphora* (Diptera) and *Locusta* (Orthoptera) (CORNWELL) 217
— *See also* Insect ocellar nerve

Osmoregulation:
and ionic regulation in *Hydra* (Lilly) 423

P

Palaemonetes varians (Leach):
Urine production by the antennal glands of (Parry) 408
Photo-kinesis:
in the ammocoete larva of the brook lamprey (Harden Jones) 492
Pigeons:
Homing orientation in, in relation to opportunity to observe the sun before release (Pratt and Thouless) 140
— An investigation of homing ability in, without previous homing experience (Pratt) 70
Polychaete:
See Nephthys
Potassium:
See Dixippus morosus

R

Rabbit:
See Blood groups
Red cells:
Electrophoretic mobility of, and their ghosts as observed with improved apparatus (Ponder and Ponder) 175
— *See also* Blood groups
Renilla:
Observations on luminescence in (Pennatulacea) (Nicol) 299
— Nervous regulation of luminescence in the sea pansy *R. köllikeri* (Nicol) 619
Respiratory physiology:
Observations on, and on the haemoglobin of the polychaete genus **Nephthys**, with special reference to **N. hombergii** (Aud. et M.-Edw.) (Jones) 110
— *See also* Woodlice
Rhodnius prolixus:
The breakdown of the thoracic gland in the adult insect (Wigglesworth) 485
— The role of the haemocytes in the growth and moulting of an insect (Hemiptera) (Wigglesworth) 649

S

Salmo trutta Linn.:
Seasonal variations in the growth rate, thyroid gland activity and food reserves of brown trout (Swift) 751
Sea anemones:
Facilitation in. IV. The quick response of *Calliactis parasitica* at high temperatures (Ross) 815
Sea pansy:
See Renilla
Sea-urchin egg:
The cortical changes on fertilization of the (Kacser) 451
— *See also* Cell surface
Sea-urchin spermatozoa:
The movement of (Gray) 775
— The propulsion of (Gray and Hancock) 802
Sialis lutaria:
The excretion and storage of ammonia by the aquatic larva of (Staddon) 84
— Ionic regulation and water balance in the aquatic larva of (Shaw) 353
— The permeability and structure of the cuticle of the aquatic larva of (Shaw) 330
Sodium:
See Dixippus morosus
Stick insect:
See Dixippus morosus

T

Teleosts:
Comparative studies of the thyroid function in, of tropical and temperate habitats (Fortune) 504
Temperature excess:
Factors affecting, of insects in sunshine (Digby) 279
Thoracic gland:
The breakdown of the, in the adult insect, *Rhodnius prolixus* (Wigglesworth) 485
Thyroid function:
See Teleosts
Thyroid gland activity:
See Salmo trutta
Transpiration:
of terrestrial isopods (Bursall) 238
Tyrosinase:
Inhibitors of (Pryor) 468

U

Urine production:
by the antennal glands of *Palaemonetes varians* (Leach) (Parry) 408

W

Water relations:
See earthworms
Waterproofing mechanism:
The cuticular, of the worker honey-bee (Glynne Jones) 95
Wax secretion:
in the cockroach (Beament) 514
Woodlice:
Cutaneous respiration in (Edney and Spencer) 256

INDEX OF AUTHORS

B

Barnes, Gordon E. The behaviour of *Anodonta cygnea* L., and its neurophysiological basis 158

Beament, J. W. L. Wax secretion in the cockroach 514

Brown, R. H. J. *See* Ramsay, Brown and Croghan

Buck, John and Keister, Margaret. Further studies of gas-filling in the insect tracheal system 681

Bursell, E. The transpiration of terrestrial isopods 238

C

Chapman, R. F. Some temperature responses of nymphs of *Locusta migratoria migratorioides* (R. & F.), with special reference to aggregation 126

Clements, A. N. The sources of energy for flight in mosquitoes 547

Cornwell, P. B. The functions of the ocelli of *Calliphora* (Diptera) and *Locusta* (Orthoptera) 217

Crisp, D. J. The behaviour of barnacle cyprids in relation to water movement over a surface 569

Croghan, P. C. *See* Ramsay, Brown and Croghan

D

Digby, Peter S. B. Factors affecting the temperature excess of insects in sunshine 279

E

Edney, E. B. and Spencer, J. O. Cutaneous respiration in woodlice 256

Ewer, D. W. *See* Pople and Ewer

F

Fortune, P. Y. Comparative studies of the thyroid function in teleosts of tropical and temperate habitats 504

G

Gray, J. The movement of sea-urchin spermatozoa 775

Gray, J. and Hancock, G. J. The propulsion of sea-urchin spermatozoa 802

H

Hamburger, Kirsten, *See* Thomsen and Hamburger

Hancock, G. J. *See* Gray and Hancock

Harris, E. J. and Martins-Ferreira, H. Membrane potentials in the muscles of the South American frog, *Leptodactylus ocellatus* 539

Holdgate, M. W. The wetting of insect cuticles by water 591

Horridge, C. A. The nerves and muscles of medusae. II. *Geryonia proboscidalis* Eschscholtz 555

—— III. A decrease in the refractory period following repeated stimulation of the muscle of *Rhizostoma pulmo* 636

—— IV. Inhibition in *Aequorea forskalea* 642

Hoyle, G. Functioning of the insect ocellar nerve 397

J

Jones, F. R. Harden. Photo-kinesis in the ammocoete larva of the brook lamprey 492

Jones, G. D. Glynne. The cuticular waterproofing mechanism of the worker honeybee 95

Jones J. D. Observations on the respiratory physiology and on the haemoglobin of the polychaete genus *Nephthys* with special reference to *N. hombergii* (Aug. et M.-Edw.) 110

Joysey, Valerie C. A study of the blood groups of the rabbit, with reference to the inheritance of three antigens, and the agglutinability of the red cells carrying them 440

K

Kacser, H. The cortical changes on fertilization of the sea-urchin egg 451

Keister, Margaret. *See* Buck and Keister

L

Lilly, Sylvia J. Osmoregulation and ionic regulation in *Hydra* 423

M

Martins-Ferreira, H. *See* Harris and Martins-Ferreira

Matthews, G. V. T. An investigation of the 'chronometer' factor in bird navigation 39

Mitchison, J. M. and Swann, M. M. The mechanical properties of the cell surface. III. The sea-urchin egg from fertilization to cleavage 734

N

Newth, D. R. and Ross, D. M. On the reaction to light of *Myxine glutinosa* L. 4

Nicol, J. A. C. Observations on luminescence in *Renilla* (Pennatulacea) 299

— Nervous regulation of luminescence in the sea pansy *Renilla kökilleri* 619

P

Parry, Gwyneth. Urine production by the antennal glands of *Palaemonetes varians* (Leach) 408

Ponder, Eric and Ponder, Ruth V. Electrophoretic mobility of red cells and their ghosts as observed with improved apparatus 175

Ponder, Ruth V. *See* Ponder and Ponder

Pople, Walter and Ewer, D. W. Studies on the myoneural physiology of Echinodermata. II. Circumoral conduction in *Cucumaria* 59

Pratt, J. G. An investigation of homing ability in pigeons without previous homing experience 70

Pratt, J. G. and Thouless, R. H. Homing orientation in pigeons in relation to opportunity to observe the sun before release 140

Pringle, J. W. S. The function of the lyriform organs of arachnids 270

Pryor, M. G. M. Inhibitors of tyrosinase 468

R

Ramsay, J. A. The excretory system of the stick insect *Dixippus morosus*, (Orthoptera, Phasmidae) 183

— The excretion of sodium, potassium and water by the Malpighian tubules of the stick insect, *Dixippus morosus* (Orthoptera, Phasmidae) 200

Ramsay, J. A., Brown, R. H. J. and Croghan, P. C. Electrometric titration of chloride in small volumes 822

Roots, Betty I. The water relations of earthworms. I. The activity of the nephridiostome cilia of *Lumbricus terrestris* L. and *Allolobophora chlorotica* Savigny, in relation to the concentration of the bathing medium 765

Ross, D. M. Facilitation in sea anemones. IV. The quick response of *Calliactis parasitica* at high temperatures 815

— *See also* Newth and Ross

S

Selman G. G. and Waddington, C. H. The mechanism of cell division in the cleavage of the newt's egg 700

Shaw, J. A simple procedure for the study of ionic regulation in small animals 321

— The permeability and structure of the cuticle of the aquatic larva of *Sialis lutaria* 330

— Ionic regulation and water balance in the aquatic larva of *Sialis lutaria* 353

— Ionic regulation in the muscle fibres of *Carcinus maenas*. I. The electrolyte composition of single fibres 383

— — II. The effect of reduced blood concentration 664

Spencer, J. O. *See* Edney and Spencer

Staddon, B. W. The excretion and storage of ammonia by the aquatic larva of *Sialis lutaria* (Neuroptera) 84

Steven, D. M. Experiments on the light sense of the hag, *Myxine glutinosa* L. 22

Swann, M. M. *See* Mitchison and Swann

Swift, D. R. Seasonal variations in the growth rate, thyroid gland activity and food reserves of brown trout (*Salmo trutta* Linn.) 751

T

Thomsen, Ellen and Hamburger, Kirsten. Oxygen consumption of castrated females of the blow-fly, *Calliphora erythrocephala* Meig. 692

Thouless, R. H. *See* Pratt and Thouless

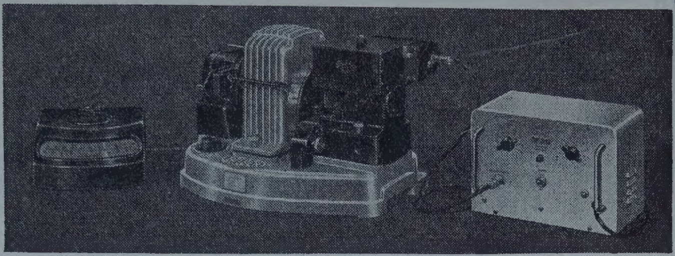
W

Waddington, C. H. *See* Selman and Waddington

Wigglesworth, V. B. The breakdown of the thoracic gland in the adult insect, *Rhodnius prolixus* 485

— The role of the haemocytes in the growth and moulting of an insect, *Rhodnius prolixus* (Hemiptera) 649

Fluorescence methods...



are being increasingly used for micro-analysis. The Spekker Fluorimeter has been redesigned to meet the demand for the very high sensitivity and accuracy necessary for this type of work. The instrument is compact, simple to operate from A.C. mains, and is provided with automatic compensation for mains voltage variations. Sets of parts are available for converting the Fluorimeter to the Spekker Absorptiometer and vice versa.

For full details write for catalogue CH 390 (*B.* 12)

Hilger & Watts Ltd., 98 St. Pancras Way, London, N.W. 1

Member of SIMA and SCIEX

Bird Navigation

G. V. T. MATTHEWS

Dr Matthews has spent several years investigating the ability of birds to return 'home' after long migrations or experimental displacement, and has himself carried out much of the critical research. This volume in the CAMBRIDGE MONOGRAPHS IN EXPERIMENTAL BIOLOGY reviews the facts and sets out the present state of knowledge. *12s. 6d. net*

The Physiology of Diapause in Arthropods

A. D. LEES

The experimental analysis of diapause has been actively pursued during the past fifteen years, and the results achieved are now in need of the integration and reappraisal. Dr Lees, whose studies on the diapause of red spider mites are among the most detailed yet made, satisfies this need in the latest volume of CAMBRIDGE MONOGRAPHS IN EXPERIMENTAL BIOLOGY. *12s. 6d. net*

CAMBRIDGE UNIVERSITY PRESS

THE JOURNAL
OF
THE LINNEAN SOCIETY OF LONDON

Vol. XLII (No. 287) **ZOOLOGY** 12 October 1955

CONTENTS

Observations on the Luminescence of Bathypelagic Crustacea Decapoda of the Bermuda Area. By Ralph Dennell.

The Polychaeta of South Africa.—Part 3. Sedentary Species from Cape Shores and Estuaries. By J. H. Day.

Spiders from Western Samoa. By B. J. Marples.

Price Twenty Shillings

LONDON:
SOLD AT THE SOCIETY'S ROOMS, BURLINGTON HOUSE
PICCADILLY, W. 1

THE QUARTERLY JOURNAL OF MICROSCOPICAL SCIENCE

Joint Editors:

C. F. A. PANTIN, Sc.D., F.R.S. JOHN R. BAKER, D.Sc.

Third Series, No. 35

Contents of Volume 96, Part 3, September, 1955

GALBRAITH, W. The Optical Measurement of Depth.

CHRISTIE, A. C. A Study of the Argentaffin (Kultschitzky) Cells in frozen-dried Tissue by Phase-Contrast Microscopy and Ultra-Violet Light.

CHRISTIE, A. C. A Study of the Kultschitzky (Argentaffin) Cell with the Electron-microscope, after Fixation by Osmium Tetroxide.

PETERS, A. Experiments on the Mechanism of Silver Staining. III. Quantitative Studies.

PETERS, A. Experiments on the Mechanism of Silver Staining. IV. Electron Microscope Studies.

PETERS, A. A General-Purpose Method of Silver Staining.

WOLMAN, M. Studies of the Impregnation of Nervous Tissue Elements. I. Impregnation of Axons and Myelin.

WOLMAN, M. Studies of the Impregnation of Nervous Tissue Elements. II. The Nature of the Compounds Responsible for the Impregnation of Axons; Practical Considerations.

WHITEAR, M. Dermal Nerve-endings in *Rana* and *Bufo*.

MURRAY, R. W. The Lateralis Organs and their Innervation in *Xenopus laevis*.

DICK, D. A. T. An Easily Made Tissue Culture Perfusion Chamber.

WARD, J. A Description of a new Zooplankton Counter.

HAYES, C. *Amoeba taylorae* n.sp.

YONGE, C. M. Adaptation to Rock Boring in *Botula* and *Lithophaga* (Lamellibranchia, Mytilidae) with a Discussion on the Evolution of this Habit.

Subscription Price per Volume of 4 Parts £4. 4s. Single Parts 30s. net

OXFORD UNIVERSITY PRESS LONDON E.C.4

Journal of the Marine Biological Association of the United Kingdom

VOL. XXXIV, NO. 3. 1955 49*s*. net

G. A. STEVEN, C. A. HOODLESS, R. S. HARRISON and F. J. WARREN. R. V. *Sarsia*: a brief description of the Marine Biological Association's new research vessel.

A. J. SOUTHWARD. On the behaviour of barnacles. I. The relation of cirral and other activities to temperature.

A. J. SOUTHWARD. On the behaviour of barnacles. II. The influence of habitat and tide-level on cirral activity.

M. R. DROOP. A suggested method for the assay of vitamin B_{12} in sea water.

D. ATKINS. The cyphonautes larvae of the Plymouth area and the metamorphosis of *Membranipora membranacea* (L.).

D. ATKINS. The ciliary feeding mechanism of the cyphonautes larva [Polyzoa Ectoprocta].

E. NAYLOR. The comparative external morphology and revised taxonomy of the British species of *Idotea*.

S. M. MARSHALL and A. P. ORR. On the biology of *Calanus finmarchicus*. VIII. Food uptake, assimilation and excretion in adult and Stage V *Calanus*.

DOUGLAS P. WILSON. The role of micro-organisms in the settlement of *Ophelia bicornis* Savigny.

N. A. HOLME. An improved 'vacuum' grab for sampling the sea-floor.

G. R. FORSTER. *Hemimycale columella* (Bowerbank): a short description and history of the species.

D. B. CARLISLE. Local variations in the colour pattern of the prawn *Leander serratus* (Pennant).

PAUL C. SILVA. The dichotomous species of *Codium* in Britain.

MARY PARKE, IRENE MANTON and B. CLARKE. Studies on marine flagellates. II. Three new species of *Chrysochromulina*.

SIR FRANCIS G. W. KNOWLES, DAVID B. CARLISLE and MARIE DUPONT-RAABE. Studies on pigment-activating substances in animals. I. The separation by paper electrophoresis of chromactivating substances in arthropods.

L. BRULL and Y. CUYPERS. Blood perfusion of the kidney of *Lophius piscatorius* L. IV. Magnesium excretion.

T. B. BAGENAL. The growth rate of the Long Rough Dab *Hippoglossoides platessoides* (Fabr.)—a correction.

ABSTRACTS OF MEMOIRS. Recording work done at the Plymouth Laboratory.

BOOK REVIEWS.

MARINE BIOLOGICAL ASSOCIATION OF THE UNITED KINGDOM.

Orders may be sent to your own bookseller or to the publishers

Cambridge University Press
Bentley House
200 *Euston Road*
London, N.W. 1

Contributors can avoid delay in the publication of their work by conforming with the following arrangements:

The Journal of Experimental Biology will publish contributions in experimental biology and comparative physiology, whether zoological or botanical. Every paper must contain a summary of the chief results of the enquiry. Authors are requested to present their work in as *concise* a form as possible. They will receive 50 copies of their papers free; additional copies may be purchased and should be ordered when the proofs are returned to the Editors.

All communications should be addressed to the Editors,

The Journal of Experimental Biology, Zoological Laboratory,
Downing Street, Cambridge, England.

MANUSCRIPTS:

MSS. should be *typewritten* (double spacing) on one side only of the paper, and the pages should be numbered. All paragraphs to be printed in small type should be indicated by adding a line at the side of the copy. *Tables* and *Legends* should be on separate sheets from the rest of the MS. and should be numbered. The place where they are to be inserted in the text should be indicated in the margin of the MS. *Bibliography* should be given under the heading of 'References' and in the following form: Surname of authors (in alphabetical order), initials, date of publication, title of paper, title of journal (abbreviated according to the *World List of Scientific Periodicals*), volume and pages of reference (first and last). In the text a reference should be quoted by the author's name and date (in brackets), and not by the numerical order of the paper in the list of references. All names of journals and books will be printed in italics.

Bodies of numerical data, too extensive for complete publication, should be summarized for discussion, and the originals deposited in the Archives established for this purpose at the British Museum (Natural History).

ILLUSTRATIONS:

(i) *Line blocks*. These should be used whenever possible. Illustrations should be pen drawings in Indian ink (*jet black and waterproof*) on smooth white Bristol board, heavy drawing paper or good quality tracing paper. Drawings must not be folded or creased in any way. *Lettering should be temporarily and lightly put in by the author in soft pencil.* Where considerable reduction is required it is essential that the drawing be of such a character as to bear such reduction. *All brush work, tinting or pencil shading is to be avoided.*

Charts and curves can often be drawn to best advantage on *graph paper ruled in pale blue*. The blue lines, while ensuring accuracy, are easily eliminated by the printer, only the blackened lines that are desired remaining.

(ii) *Text half-tone blocks*. These are suitable for illustrations involving brushwork, or in which the depth of shading is an essential feature. They can be used for such things as oscillograph records and some photographs can be reproduced in this way, a good glossy bromide print being required. Illustrations should be gummed on *white* card, grouped and numbered as they are to appear in print. All lettering should be shown in position on a covering sheet of transparent paper.

(iii) *Plates*. Plates should be used only for illustrations, such as photomicrographs, in which the most accurate reproduction of fine detail is called for. Plates are expensive and the Editors may require an author to defray the cost of plates which in their opinion are not essential. The photographs making up the plate should be gummed on *white* card, grouped and numbered as they are to appear in print. Exclusive of margin the plate figures should not cover, when reduced, an area greater than 7½ in. in length × 5 in. in width when ready for reproduction as a single plate, or 7½ in. × 11½ in. in the case of double plates. All lettering should be shown in position on a covering sheet of transparent paper.

Authors are asked not to submit sheets of illustrations which are more than foolscap size; or, if this cannot be avoided, to include photographic reductions for the convenience of referees.

PROOFSHEETS:

Authors will receive one set of slip proof for correction and return to the Editors. A page proof will also be sent if the slip proof is marked by the author 'Revise'. An allowance of ten shillings per sheet of sixteen pages will be made for alterations apart from printer's errors. Authors may be charged for any excess over this average.

THE SOCIETY FOR EXPERIMENTAL BIOLOGY

The Society exists for the promotion of experimental biology. It holds Conferences, generally four times a year, at which the results of experimental investigations are presented and discussed. *The Journal of Experimental Biology* is the medium of publication of the Society. Enquiries about membership of the Society should be addressed to the Secretaries:

Dr J. W. L. BEAMENT, Department of Zoology, Cambridge

Dr R. SCOTT RUSSELL, Department of Agriculture, Oxford

PRINTED IN GREAT BRITAIN AT THE UNIVERSITY PRESS, CAMBRIDGE
(BROOKE CRUTCHLEY, UNIVERSITY PRINTER)